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INCLUSÃO EM CICLODEXTRINAS PARA ESTABILIZAR HÓSPEDES MULTI-COMPONENTE DE ORIGEM VEGETAL

CYCLODEXTRIN INCLUSION TO STABILISE MULTICOMPONENT GUESTS OF PLANT ORIGIN

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Inclusão em Ciclodextrinas para Estabilizar Hóspedes Multi-componente de Origem Vegetal

Cyclodextrins Inclusion to Stabilise Multicomponent Guests of Plant Origin

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Palavras chave Ciclodextrinas, complexos de inclusão, extrato de gengibre, gingerois, óleo essencial de *Cistus ladanifer*

Resumo

A inclusão em ciclodextrinas (CDs) de hóspedes multi-componente, tais como óleos essenciais e extratos de plantas, é um tema atual e útil para uma série de aplicações nas indústrias alimentar, cosmética e farmacêutica. No presente trabalho foram estudados dois destes sistemas, tendo por hóspedes o óleo essencial de *Cistus ladanifer* e uma mistura de gingerois extraídos de rizoma fresco de gengibre.

A composição do óleo essencial de *C. ladanifer* foi estudada por GC-MS, tendo-se identificado 94,3% dos seus componentes e determinado a massa molecular aproximada em 143,7 $g.mol^{-1}$. O óleo foi usado para formar complexos de inclusão com as ciclodextrinas beta e gama. A identificação dos componentes do óleo incluídos preferencialmente em cada CD foi feita por extração com clorofórmio e análise por GC-MS, tendo-se observado inclusão preferencial de compostos de maior peso molecular na ciclodextrina beta, enquanto a ciclodextrina gama incluiu compostos de menor peso molecular. Os complexos de inclusão foram analisados no estado sólido por espectroscopia de infravermelho (FTIR), $\{^1H\}$ ^{13}C CP-MAS RMN e difração de raios X de pós (PXRD), postulando-se empacotamento em canal para ambos os complexos.

A mistura de gingerois, obtida a partir de gengibre fresco por maceração em acetona e purificação em coluna, foi analisada por ^1H RMN e espectrometria de massa (ESI-QTOF), contendo 54,05 % de 6-gingerol, 19,45% de 8-gingerol e 26,5 % de 10-gingerol, a que corresponde uma massa molecular de 314,7 $g.mol^{-1}$. O complexo γ-CD·gingerois, obtido por co-precipitação, foi caracterizado por FTIR, $\{^1H\}$ ^{13}C CP-MAS RMN, DSC e PXRD. Também neste caso foi observado o empacotamento em canal. Por um ajuntamento segundo Pawley, foi possível refinar os parâmetros de célula em a = b = 23,886(3) Å e c = 23,356(3) Å (tetragonal). A atividade antioxidante de γ-CD·gingerois foi estudada pelo ensaio de proteção do β-caroteno, tendo-se obtido resultados similares aos dos gingerois não incluídos. Os gingerois e o γ-CD·gingerois foram usados para preparar iogurte fortificado com 1% (m/m) de gingerol (ou equivalente de complexo) tendo-se verificado que o complexo é mais facilmente disperso na matriz do que os gingerois não encapsulados. A cor do iogurte fortificado com γ-CD·gingerois apresentou-se mais semelhante à do iogurte simples enquanto para o iogurte com gingerois registaram maiores diferenças. Os iogurtes fortificados foram ainda estudados quanto à durabilidade, não se tendo observado alterações de pH nem aparecimento de odores desagradáveis durante quatro semanas, enquanto no iogurte simples a formação de odor se iniciou entre a segunda e a terceira semana. A atividade antioxidante dos iogurtes fortificados medida pelo método de ABTS foi superior à do controlo, sendo a condição mais promissora verificada para a amostra com gingerois. Estes resultados sugerem que a matriz interfere com a atividade antioxidante de γ-CD·gingerois.

Key words Cyclodextrins, inclusion complexes, ginger rhizome extract, gingerols, *Cistus ladanifer* essential oil

Abstract

Cyclodextrin inclusion of multi-component guests such as essential oils and plant extracts is a current topic of research. These systems are usefull for a number of applications in food, cosmetic and pharmaceutical industries. The present work focus on two of these systems, having as guests *Cistus ladanifer* essential oil and a mixture of gingerols obtained from fresh ginger rhizome.

The *C. ladanifer* essential oil composition was elucidated by GC-MS, which allowed identifying 94.3 % of the components and to establish the approximate Mw at 143.7 $g.mol^{-1}$. The oil was subsequently included into beta and gamma cyclodextrins (β and γ-CDs) by co-precipitation. Identification of the included components of the oil was done by chloroform extraction followed by GC-MS analysis. β-CD preferentially included compounds of higher molecular weight, whereas γ-CD included lower molecular weight compounds. Solid state analysis of the inclusion complexes comprised infrared spectroscopy (FTIR), $\{^1H\}$ ^{13}C CP-MAS RMN and powder X-ray diffraction (PXRD) that suggests the occurrence of channel packing for both.

Gingerols were obtained from fresh ginger by maceration in isopropanone followed by column chromatography. The product was analysed by ^1H RMN and mass spectrometry (ESI-QTOF), revealing a composition of 54.05 % 6-gingerol, 19.45 % 8-gingerol and 26.5 % 10-gingerol, and a corresponding Mw of 314.7 $g.mol^{-1}$. The γ-CD·gingerols complex was obtained by co-precipitation and characterized by FTIR, $\{^1H\}$ ^{13}C CP-MAS RMN, DSC and PXRD. It presented the typical γ-CD complexes packing in the form of infinite channels. PXRD data was further treated with a Pawley extraction allowing to identify a tetragonal unit cell with the parameters refined at a = b = 23.886(3) Å e c = 23.356(3) Å. The antioxidant activity of γ-CD·gingerois and free gingerols, as evaluated by the β-carotene bleaching assay, showed similar potencies. Free gingerois and the complex of γ-CD·gingerols were employed in fortification of yoghurt, at a concentration of 1% (m/m) of gingerol (or its equivalents mass for the complex). A better dispersion into the matrix was observed for the γ-CD·gingerols–fortified yogurts in comparison with gingerols-fortified samples. The colour of the yoghurts fortified with the complex was almost similar to that of plain yoghurt, whereas those fortified with free gingerols had more colour variation in regard to plain yoghurt. The storage stability of fortified yoghurts was evaluated through pH monitoring and the formation of malodours. No changes in pH or malodours were observed for four weeks. In turn, a malodour in simple yoghurt was noticed starting from the second to the third week of storage. The antioxidant activity of yoghurts, as measured by the ABTS assay, revealed a higher antiradical action for gingerols-fortified and γ-CD·gingerols–fortified yogurts when compared to that of plain yogurts, with the most promising results being registered for the gingerols-fortified samples. These particular results suggest that food matrix might interfering the antioxidant activity of γ-CD·gingerols.

Lista de Abreviaturas / List of Abbreviations

Lista de Compostos e Enzimas / List of Compounds and Enzymes

Abreviatura	English	Português
5-LOX	5-Lipoxygenase	5-Lipoxigenase
$ABTS^{\cdot+}$	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid	Ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfónico)
BHA	Butylated Hydroyanisole	Hidroxianisol Butilado
BHT	Butylated Hydroxytoluene	Hidroxitolueno Butilato
CA	Cycloamylase	Cicloamilase
CAT	Catalase	Catalase
CD	Cyclodextrin	Ciclodextrina
CGTase	Cyclodextringlycosyltranferase	Ciclodextringlicosiltransferase
COX	Cyclooxygenase	Ciclooxigenase
DMSO	Dimethylsulfoxide	Dimetilsulfoxido
Dimeb	Dimethylated β-Cyclodextrin	β-Ciclodextrina Dimetilada
DNA	Deoxyribonucleic Acid	Ácido Desoxirribonucleico
EO	Essential Oil	Óleo Essencial
GPx	Glutathione Peroxidase	Glutationa Peroxidase
GR	Glutathione Reductase	Glutationa Redutase
GSH	Total Glutathione	Glutationa Total
GST	Glutatione S-Transferase	Glutationa S-Transferase
HCl	Hydrochloric Acid	Ácido Clorídrico
HPβCD	(2-)Hydroxypropyl-β-Cyclodextrin	(2-)Hidroxipropil-β-Ciclodextrina
HPγCD	(2-)Hydroxypropyl-γ-Cyclodextrin	(2-)Hidroxipropil-γ-Ciclodextrina
IC	Inclusion Complexes	Complexos de Inclusão
IL	Interleukin	Interleucina
LR-CDs	Large Ring Cyclodextrins	Ciclodextrinas de Anel Largo
MDA	Malondialdehyde	Manoldialdeído
NaCl	Sodium Chloride	Cloreto de Sódio
NaOH	Sodium Hydroxide	Hidróxido de Sódio
NADP	Nicotinamide Adenine Dinucleotide Phosphate	Nicotinamida Adenina Dinucleótido Fosfato
NSAID	Non-Steroidal Anti-Inflammatory Drug	Drogas Não-Esteróis Anti-Inflamatórias
PG synthetase	Prostaglandin Synthetase	Prostaglandina Sintetase
PGE_2	Prostaglandin E_2	Prostaglandina E_2
Rameb	Randomly Methylated β-CD	β-Ciclodextrina Aleatoriamente Metilada
SOD	Superoxide Dismutase	Superóxido Dismutase
TXB_2	Thromboxane B_2	Tromboxano B_2
Trimeb	Trimethylated β-Cyclodextrin	β-Ciclodextrina Trimetilada
TNF-α	Tumor Necrosis Factor-α	Fator de Necrose Tumoral-α
VE	Vitamin E	Vitamina E

Lista de Abreviaturas de Nomes de Técnicas e Ensaios Analíticos /
List of Abbreviations of Techniques and Assays

Abreviatura	English	Português
^{13}C NMR CP/MAS	Carbon-13 Nuclear Magnetic Resonance with Cross Polarization and Magic Angle Spinning	Ressonância Magnética Nuclear de Carbono 13 com Polarização Cruzada e Rotação Segundo o Angulo Mágico
DPPH	1,1-Diphenyl-2-Picrylhydrazyl Radical	Radical 1,1'-Difenil-2-Picril-Hidrazilo
DSC	Differential Scanning Calorimetry	Análise de Calorimetria Diferencial
DW	Dry Weight	Peso Seco
EC_{50}	Half Maximal Effective Concentration	Concentração com 50% de Eficácia
ELISA	Enzyme-Linked Immunosorbent Assay	Ensaio de Imunossorvente Ligado a Enzimas
ES / ESI	ElectroSpray Ionization (for MS)	Ionização por Electrospray (usada em MS)
FCA	Freund's Complete Adjuvants	Completos de Freund Adjuvantes
FRAP	Ferric-Reducing Ability Power	Poder de Redução do Ferro
FTIR	Fourier Transformed Infrared Vibrational Spectroscopy	Espetroscopia Vibracional de Infravermelho com recurso à Transformada de Fourier
GAE	Gallic Acid Equivalents	Equivalentes de Ácido Gálico
GC	Gas Chromatography	Cromatografia em Fase Gasosa
GC-sniffing	Gas Chromatography – Sniffing	Cromatografia de Gás por Cheiro
HS-SPME	Headspace – Solid Phase Microextraction	Microextracção de Fase Sólida por Exposição a Vapor
HPLC	High Performance Liquid Cromatography	Cromatografia Líquida de Alto Desempenho
MIC	Minimum Inhibitory Concentration	Concentração Inibitória Mínima
MS	Mass Spectrometry	Espectrometria de Massa
ORAC	Oxygen Radical Absorbance Capacity	Capacidade de Absorção do Radical Oxigénio
PA	Purity Analytical	Pureza Analítica
PXRD	Powder X-Ray Diffraction	Difração de Raios-X de Pós
QE	Quercitin Equivalents	Equivalentes de Quercetina
SCFE-CO_2	Supercritical Fluid Carbon Dioxide Extraction	Extração por Dióxido de Carbono Supercrítico
TBARS	Thiobarbituric Acid-Reactive Substance	Substância Reativa de Ácido Tiobarbiturico
TE	Trolox Equivalents	Equivalentes de Trolox
TEAC	Trolox Equivalent Antioxidant Capacity	Capacidade Antioxidante em Equivalentes de Trolox
UV	Ultraviolet [Spectroscopy]	[Espectroscopia de] Ultravioleta

Lista de Acrónimos de Entidades Reguladoras e Conceitos Associados / Acronyms of Regulatory Identities and Related Concepts

Acrónimo	English	Português
ADI	Acceptable Daily Intake	Dose Diária de Consumo Aceitável
EC	European Commission	Comissão Europeia
FD&C Act	Federal Food, Drug and Cosmetic Act	Ato Federal de Proteção a Alimentos, Remédios e Cosmética (dos Estados Unidos)
FDA	Food and Drug Organization	Administração de Alimentos e Remédios
FSANZ	Food Standards Australia and New Zealand	Entidade de Padronização de Alimentos da Austrália e Nova Zelândia
GRAS	Generally Regarded as Safe	Considerado em Geral Seguro
IUPAC	International Union of Pure and Applied Chemistry	União Internacional de Química Pura e Aplicada
WHO	World Health Organization	Organização Mundial de Saúde

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1. Introduction

1.1. Cyclodextrins, from Enzymatic Digest Products to Inclusion Complexes

1.1.1. From Early Discovery till Today: the History and Production of Cyclodextrins

Cyclodextrins were first described by Villiers, in 1891[1]. Villiers isolated a substance from bacterial digest of starch that was resistant to acid hydrolysis and did not show reducing properties. This was called "cellulosine", because it had resemblances to cellulose. Later on, Schardinger studied *bacillus* isolated from cereal based foodstuff, which had the ability to form "crystalline dextrin" – the name given by Schardinger to cyclodextrins[2]. The bacteria fed on an aqueous suspension containing only starch to produce a clear solution in 24h at 50 ºC, enabling the easy formation crystals of cyclodextrins as the water evaporated. In a follow-up study, Schardinger perfected the crystallisation process and isolated the bacterial strain that affords the cyclodextrins, naming it *Bacillus macerans*[3], being now referred as *Bacillus firmus* after a taxonomical revision. Nowadays, it is known that the production of cyclodextrins relies on the action of the enzyme responsible for starch fragmentation and cyclisation, named cyclodextringlycosyltranferase (CGTase)[4].

Following the studies of Villiers and Schardinger, other scholars continued to work on cyclodextrins, but, until 1935, this field of research underwent a "period of doubt"[5]. This designation was given due to doubts regarding the purity of the dextrins used, as well as the structure, which had not yet been well established. From 1935 to 1950, the investigation with cyclodextrins reached a "period of maturity" and a consensus on the nomenclature was attempted. It was Friedrich Cramer that suggested the nomenclature and the abbreviation for cyclodextrins, in his doctoral thesis, in 1949, even though it remained a subject of debate until the 1990s[5]. Alterations involved the use of the Greek alphabet depending on the number of glucose units and the use of CD abbreviation for cyclodextrin. This way, the main and most common products of the CGTase enzyme, named native cyclodextrins and having six, seven or eight α-D-glucose units, are named alpha, beta and gamma (α-CD, β-CD and γ-CD), respectively.

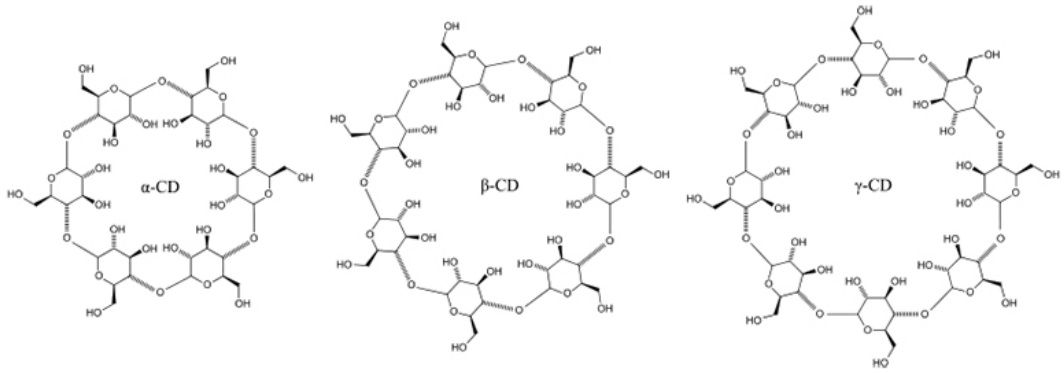

Figure 1 – Schematic representation of the three most abundant cyclodextrins: α-, β- and γ-CDs (left to right).

Cyclodextrins with higher number of glucose units, referred to as large ring cyclodextrins (LR-CDs), are less common, although some may also be formed in trace amounts by action of CGTase, namely rings with nine (δ-CD), ten (ε-CD) or more glucose units[6]. Currently, LR-CDs with up to 54 glucose units can be produced using a different enzyme, amylomaltase, and pea starch as the substrate[7].

1.1.2. Structure and Properties of Cyclodextrins

In cyclodextrins, the α-(1, 4) glucose units are linked at $C1_{(n)}$-$O4_{(n-1)}$-$C4_{(n-1)}$, where *n* is the number of a glucose unit. This connection and the bond between C6-O6 of the glucose units are the only conformational freedom points of CDs[8]. Thus, there is possibility of slight variation in the angles and torsion these bonds, resulting in non-equivalent carbons in the different monomer units. The conformation of $^4C_1$ α-D-glucopyranose units causes all secondary hydroxyl groups to be positioned at the wider rim of the ring and the primary ones to face the narrower rim[9]. The cavity is lined with H_3 and H_5 hydrogens and the glycosidic oxygen bridges, thus having hydrophobic character.

The height of all native cyclodextrins is constant – 7.9 Å[9] – and only the diameter changes as a result of them having different number of glucose units. The differences in their properties arise this precisely from the different number of glucose units, implying different diameters and cavity volumes (Table 1). These two characteristics make it possible to form inclusion complexes with hydrophobic molecules having adequate shape and polarity to be encapsulated. Note also that in this process it is possible to encapsulate several guest molecules. The inclusion complexation process will be detailed in subsection 1.1.4.

Table 1 – Physico-chemical and structural properties of native cyclodextrins. Adapted from J. Szejtli[9].

Property	α-CD	β-CD	γ-CD
Number of glucose units	6	7	8
Molecular weight, g/mol	972	1135	1297
Solubility in water at room temperature, g/100 mL	14.5	1.85	23.2
Diameter of the cavity, Å	4.7 – 5.3	6.0 – 6.5	7.5 – 8.3
Diameter of the outer periphery, Å	14.6 ± 0.4	15.4 ± 0.4	17.5 ± 0.4
Approximated volume of cavity, Å^3	174	262	427

In particular, β-CD has a much lower aqueous solubility than the other two native CDs, which results from the intramolecular hydrogen bonds formed between the secondary hydroxyl groups of all its glucose units[10]. The chirality of the glucose units of β-CD induces energy differences between geometries with different orientation of the co-operativity of hydrogen bonds. These intramolecular hydrogen bonds are homodromic, forming a closed, monodirectional circle which contributes to stabilize the macrocyclic conformation of β-CD, leading to a lower solubility.

Native cyclodextrins can be chemically modified to modulate their physico-chemical and biological properties – cavity depth, reactivity and polarity. These reactions involve the hydroxyls of the primary and/or the secondary rims[11]. Modified cyclodextrins can be more water soluble and useful as enzyme mimics, because the substitute groups that are linked to the cyclodextrin can act through molecular recognition. The use of modified cyclodextrins increases their application range, because, in addition to acting as delivery systems, they increase bioavailability and activity of drug, as they are more soluble than their native ones.

1.1.3. Safety of Cyclodextrins

α-CD is approved by the WHO/FAO Joint Expert Committee on Food Additives (JEFCA) as a food additive since 2002[12] and as a dietary fiber since 2006[13], in both uses having an acceptable daily intake (ADI) "not specified", which means that it may be used in food in unrestricted quantities. In the United States, it has been the last native cyclodextrin to be awarded the GRAS status in 2004[14]. β-CD is the only native cyclodextrin with consumption restrictions. In 2001, β-CD was classified as GRAS[15], but it has a daily intake limit (ADI) of 5 mg/kg[16]. Both α- and β-CDs are indigestible *in vivo*, while γ-CD is considered a slowly digestible carbohydrate[17]. For this reason, γ-CD was the first native CD

to be approved for uses in foodstuff in countries outside of Japan. In 1999, the JEFCA approved γ-CD as a food additive[18] and it received the GRAS status in the following year[19]. Nowadays, β-CD is still listed by its food additive number – E459[20].

In in Australia, New Zealand and Europe, α- and γ-CDs are denominated as *"Novel Food"*, which means that the use of their additive number is not required and they can be listed by their name[21].The Food Standards Australia and New Zealand (FSANZ) has accepted α- and γ-CD as novel food in 2004[22] and in 2003[23], respectively. In Europe, the use of the term novel food for cyclodextrins was late applied. α-CD was accepted as novel food in 2008[24] and γ-CD has this nomination since 2012[25]. In Japan, native CDs are used without restriction and have made their way into a very large number of products[26].

Chemically modified cyclodextrins are also subject to regulatory control and safety approval. In the European Union, this cyclodextrins are regulated as excipients and their use is approved by the European Medicines Agency according to the delivery route. 2-hydroxypropyl-β-cyclodextrin (HPβCD) is considered safe for consumption, although there are references to some biochemical changes in the body, namely the increase of some plasma levels of enzymes, on test animals (rats, mice and dogs), and diarrhea, in humans[27], while FDA only approved its use for topical route[28].

In Canada, native cyclodextrins are classified as synthetic duplicates of natural products and they have been approved as 'natural medicinal ingredients' (NMIs) since 2011 and 'natural health products' (NHPs) with the function of 'controlled release vehicle' and currently they are registered as emulsifying, sequestering, stabilising and encapsulating agents. HPβCD is registered as an emulsion stabiliser and sequestering agent and DIMEB as a sequestering, stabilising and encapsulating agent[29].

In addition to being able to be used in the formulation of certain foodstuffs with stabilizing and emulsifying properties, the cyclodextrins may be present in cosmetic and daily care products. It is known from food applications that CDs are not dangerous for human consumption (the gastrointestinal mucosa does not undergo any kind of irritation reaction when in contact with foods containing cyclodextrins)[30]. However, the outer membrane of the skin has a quite different microbial flora, whereby the topical reactions must be evaluated considering using CDs in cosmetics.

Approved cosmetic ingredients are reviewed by the Cosmetic Ingredient Review Expert Panel, an independent, non-profit scientific body established in 1976 that regularly

examines and establishes the safety of ingredients[31]. This entity has approved native CDs, HPβCD, HEβCD, Rameb and mixtures of native CDs with laurate and hydroxypropyltrimonium chloride as safe ingredients for cosmetics[32].

For dermal products, native cyclodextrins are considered safe at concentrations up to 0.1%, as well as HPβCD that is also considered safe without mentioned dose restriction[33]. The methylated-CD 2-hydroxypropyl-γ-cyclodextrin (HPγCD) was approved as an inactive ingredient by FDA, but it is limited to the topical route and to a maximal concentration of 1.5% (w/v)[28], respectively.

A comparative study with native and chemically modified CDs, namely β-CD, γ-CD, Rameb, Dimeb, Trimeb, HPβCD and HPγCD, using a bioassay named corneoxenometry in healthy volunteers showed no harmful effects on the skin[34]. This bioassay measures the compatibility of xenobiotics with the *stratum corneum* and the manifestation of irritation or any alteration in the *stratum corneum* structure. The corneoxenometry suggested that all cyclodextrins tested are well tolerated by the skin.

1.1.4. Inclusion Complexes – the Supramolecular Assembly of Cyclodextrins

In aqueous solution, the cavity of cyclodextrins is occupied by water molecules, which, due to their polarity, are energetically unfavoured and thus easily replaced by a less polar guest molecule. This process is denominated molecular encapsulation and it is not a chemical reaction[9,35]. In particular, the hydrophobic or Van der Waals forces keep the guest included in the cyclodextrin, and the various cyclodextrins interact via hydrogen bonds. Typically, the formation of an inclusion complex causes the decrease of cyclodextrins ring strain and leads to a more stable and lower energy state. A good binding between host and guest depends on the volume of the cavity of the cyclodextrin and the size of the guest as well as on specific local interactions between surface atoms. Even though the inclusion complex is a stable adduct, a dynamic equilibrium between cyclodextrin and guest is achieved in the free and encapsulated forms, when the complex is in solution.

Usually, water is the solvent of choice to solubilise CDs. Unfortunately, as the guest molecule is hydrophobic, this molecule needs to be dissolved with a co-solvent. In general, the host:guest ration is 1:1, but other ratios can occur such as 2:1, 1:2, 2:2 and even multiple stoichiometries, though these are quite rare[9]. In the inclusion complex, the guest molecules

are wrapped individually or in pairs (depending on the stoichiometry of inclusion) by the CD cavity and therefore their behaviour is dramatically altered.

The guest molecule can be solid, liquid or gaseous, and can comport a variety of chemical structures – straight or branched compounds – and families – aliphatics, aldehydes, ketones, alcohols, organic acids, fatty acids, aromatics, gases, halogens, oxyacids and amines[36]. The great variety of compounds that can act as guest allows inclusion complexes of cyclodextrins to be applied in products that generally do not have CDs in their composition and this use increases the quality of the product.

In the literature, there are already several essential oil encapsulated[37]. Table 2 presents several essential oils inclusion complexes using β-CD, as well as HPβCD, with the effect observed. The use of these cyclodextrins is due to the fact that the cavity has the size indicated for the complexation. On the other hand, the use of the modified cyclodextrin allows a more soluble final product to be obtained in water, since the solubility of HPβCD is higher than that of β-CD.

Table 2 – Essential oil (EO) inclusion complexes in β-CD and HPβCD.

Cyclodextrin	Guest – Essential Oil	Ratio	Observation(s)/Effect(s)	Ref
β-CD	*Cinnamonum verum*	1:1	Mask taste, increase solubility and antimicrobial action	[38]
	Coriandrum sativum	dns	Same antioxidant activity than guest alone	[39]
	Eugenia caryophyllata	1:1	Mask taste, increase solubility and antimicrobial action.	[38]
	Lavandula angustifolia	dns	Formation of controlled release systems for the delivery of essential oil used as ambient odours.	[40]
	Lavandula spica	dns	Increase of durability of volatility fragrance compounds on textiles.	[41]
	Mentha piperita	1:1	Controlled release of aroma compounds.	[42]
	Satureja montana	1:1	Increase solubility, antioxidant, antibacterial and antifungal actions.	[43]
	Thymus vulgaris	1:1	Increase solubility, improve antimicrobial action, by decreasing the needed concentration, protect EOs from degradation and convert into a powder.	[44]
HPβCD	*Lavandula viridis*	dns	Antioxidant activity of the EO/HPβCD complexes were more stable over 30 days, at 4ºC than comparative β-CD complexes.	[45]
	Lavandula pedunculata	dns		
	Thymus lotocephalus	dns		
	Lippia gracilis	dns	Possible application at the breeding site of the dengue mosquito.	[46]

Note: dns – data not showed.

Cyclodextrins may further have the ability to select the type of molecule to include, i.e., the hydrophobic cavity may be selective in behavior. Testing different cyclodextrins permit determining which one forms the strongest inclusion complex. This procedure was performed for the essential oil of basil and tarragon[47].

1.1.5. Advantages of Cyclodextrins and Inclusion Complexes Application

The solubilising, stabilising and protecting actions of both native and chemically modified CDs on the included guests make them useful for several applications and are currently ubiquitous in a variety of products, including foodstuff[48], pharmaceuticals[49] and cosmetics[50]. There is a great variety of possible guest molecules, being preferentially apolar, low solubility drugs, volatile compounds warranting stabilization or light-sensitive compounds.

Both in the singular form and as inclusion complex, the native and modified cyclodextrins are applied in the various areas of product development. Inclusion of aromatic molecules is one of the earliest applications of cyclodextrins. These CDs remain among the most widely used, as demonstrated by the large variety available from commercial sources. The hydrophilicity, small size and adequate geometry of the fragrance molecules make them excellent guests for cyclodextrins.

CD are presently used with a variety of functions, from solubilising agents to formulation helpers. The most obvious advantage of using ICs is the increase in the solubility of guests and they can also stabilized labile guests against the degradative effects of oxidation, visible or UV light and heat[50,51]. They can provide controlled release of active ingredients, reduce or prevent skin and gastric irritation and prevent interactions between formulation ingredients, because they allow the physical isolation of incompatible compounds, avoiding undesirable interactions that would alter the properties of the product.

1.1.5.1. Application of Cyclodextrins in Foodstuff

CDs are used as preservatives in a variety of spices, flavors, tea, vinegar and seeds, or as emulsifying enhancers in whipped creams, mayonnaise and dressings. With the advancement of technological developments, CDs and their ICs are more and more employed in the food industry as they help increase shelf life and allow microbiological protection.

β-CD can be used as a partial fat substitute[52]. The effect of β-CD on the flavour-release profiles of non-fat flavoured milks and yogurts was analysed by headspace gas chromatography to reveal that these products mimic quite well their corresponding higher-fat-content counterparts. Then, a sensory evaluation study was carried out yogurts and salad dressings. The panel found that the addition of β-CD to the non-fat yogurt did not provide it with the flavour and texture matching those of the fat-containing yogurts, but when evaluating salad dressings (Italian- and ranch-style) they found that flavour profiles of non-fat dressings were indeed more similar to the corresponding full-fat products. β-CD polymers are used to process milk[53,54] and butter[55,56], by sequestering cholesterol that gets easily included in the cyclodextrin' cavity and affording free- or low-cholesterol foods. On the other hand, γ-CD is commonly incorporated into the dough of baked products[48]. Cyclodextrin in breads, rolls, muffins and bagels is labelled under the category of preservative. An example is HealthyLife's whole wheat bread (by Lewis Bakeries Inc.)[57].

Cyclodextrins are also applied in beverages. α-CD permits an easy incorporation of a desired molecule without causing changes in the appearance of the beverage, as α-CD is water soluble and its inclusion complexes dissolve to from crystalline solutions[48]. β-CD allows masking undesired flavours, for instance, the bitterness of ginseng-based drinks[58]. Addition of β-CD with or without the combination with γ-CD was shown to reduce the bitter flavour of ginseng by roughly one half, when incorporated into a ginseng-based energy drink formulation.

Among commercially available inclusion complexes, γ-CD·curcumin is already sold by Wacker Chemie and it has approved use for dietary supplement[59]. There are also patents relating to food products with cyclodextrins. The application of γ-CD in a drink was patented[60] as well as a nutritional product containing γ-CD in a fraction of 5 to 100 wt.% of the total carbohydrates, designed for women with gestational diabetes and claimed to reduce

the postprandial insulin secretion in order to normalize blood glucose levels and prevent night-time hypoglycaemia[61].

Upon ingestion, native CDs are practically not absorbed, because of their bulky and hydrophilic nature. Absorption by passive diffusion is neglectable[48]. α-CD and β-CD are resistant to stomach acid and to salivary and pancreatic enzyme digestion, while γ-CD is digested partly by amylases in the large intestine[62]. In Windsar rats, α-CD ring opening was shown to occur by action of the gut flora (microbial enzymes) to form linear malto-oligosaccharides[63,64]. These products are hydrolyzed and fermented by routes similar to those of resistant starch. Consequences of these fermentation processes, bloating, include flatulence, nausea and diarrhea which were observed in rats and beagle subjected to regular consumption of α-CD (between 4 and 13 weeks)[65,66]. These symptoms were also reported by about 30% of the human subjects participating in the study on the effects of ingestion of cooked rice with α-CD[67].

γ-CD, in turn, has a good gastrointestinal tolerability. A double-blind placebo-controlled, cross-over, randomized study with 24 volunteers ingesting yogurt with γ-CD (8% $^{m}/_{m}$) revealed that the yogurt was well tolerated and did not cause any gastrointestinal discomfort[68]. In other study, consumption 25 g of γ-CD dissolved into a drink of 240 mL caused no significant side effects in the 24 hours after ingestion[69].

α-CD can associated with lipid molecules, forming inclusion complexes which leads to a reduction in the problems associated with the passage of free fat through the gastrointestinal tract (steatorrhea and intestinal incontinence) and to an improvement in the lipid profile in the blood[70]. In healthy humans, the consume of α-CD in a meal provides a reduction in postprandial triglycerides levels[71], in total and LDL cholesterol (LDL = low-density lipoprotein) and in body weight[70]. In rats, a diet with 21% milk fat and 2.1% of α-CD showed reduced seric proatherogenic lipoproteins and trans-fatty acids[72].

Cyclodextrins also act on glycemic index. α-CD is already patented in bread, that allows the decrease for glycemic index, as with the consumption of this bread, the blood glucose concentration did not tend to rise above 110 mg/dL and the blood insulin concentration did not increase above 65 μUI/mL, instead of 120 μUI/mL observed on plain bread consumption[73]. Relatively to γ-CD, in a study with 32 healthy adults (double-masked, randomized, crossover design), the intake of a drink containing 25 g of γ-CD on 240 mL of

drink was shown to effectively lower post-prandial glycemia and insulinemia when compared to an equivalent maltodextrin-containing drink[74].

1.1.5.2. Application of Cyclodextrins in Cosmetics

The largest organ in the human body, the skin, acts as tough, flexible, structural barrier to invasion. It has its own flora, that may vary under the influence of multiple factors such as pH, temperature, presence of sebum, fatty acids and salt[75]. This structured barrier can suffer a break and cosmetic products are important to keep it clean, structured and safe. Therefore, the number one priority for cosmetic and personal care products companies and drugs and pharmaceutical companies is consumer safety. The products conceived to be applied topically on the skin can be classified as cosmetics or topical drugs.

Cosmetics, known as products that are intended to clean or to enhance beauty, are not regulated by the United States Food and Drug Administration (FDA)[76], only requiring the FDA approval in case of having colour additives. On the other hand, sunscreens, antiperspirants, diapers ointments and treatments for dandruff or acne are cosmetic drugs and they require both cosmetic and drug approval. Cosmetic are regulated by the Federal Food, Drug, and Cosmetic Act (FD&C Act) and it is required that "every cosmetic and personal care product and its ingredients be substantiated for safety before going to the market, and that they contain no prohibited ingredients"[77]. This means that ultimately the safety of cosmetics is the responsibility of companies. Allergies and rashes can be reported to the FD&C Act, but, as this depends on the consumer itself, it is not mandatory for the company to assume the occurrence.

From 2000 until the present day, cyclodextrins are gaining more relevance as technological solutions for dermopharmaceutical and cosmetic products[78]. In the cosmetics, CDs were used as masking agents for components with unpleasant aroma and stabilising active ingredients. Generally, CDs are listed in the ingredients as *cyclodextrin* and they are found in a variety of anti-aging products that contain potent, yet unstable, antioxidant ingredients, such as anti-aging treatments, facial moisturisers or lotions, sunscreens, cleansers, eye creams, deodorants and self-tanners.

Cyclodextrins, either pure or in the form of inclusion complexes, are widely used in cosmetic products. As cosmetics are generally placed at room temperature, are daily used by healthy people over long periods and are mainly composed of oleaginous raw materials,

ultraviolet absorbents, antioxidants, colouring materials and fragrance materials, among others[79], most of them easily perishable, when used in the free form, CDs help stabilise components, in other to ensure the constancy, security and safety of the product. Even though it is known that cyclodextrins are used on the formulation of skin products, the quantity of cyclodextrins applied is not really known, because there are no references of defined quantities in patent documents. At the same time, patent documents do not specify for which purpose cyclodextrins are applied in the product.

The delivery of drugs through the skin may be enhanced by improving the release of drug from the transdermal pharmaceutical preparation bases, the increasing of the flux of drug through the skin or by the retention of drug in the skin, the increasing in topical or localized skin delivery or tissue targeting of drug or a combination of the previous factors. Parent cyclodextrins and hydrophilic derivatives can affect the permeability of drugs through the skin, the bioconversion of drugs in the viable skin and the topical irritation caused by drugs[80,81]. The parent cyclodextrins and the modified HPβCD are not able to induce disruption of the stratum *corneum*[82], as they are too large to be able to penetrate deeply[83,84], which implicates that they are used a as a vehicle for the guest lipophilic molecules that can compete with the membrane constituents. Table 3 shows the studied effect of native and modified cyclodextrins on the permeability of compounds through the skin, both *in vitro* and *in vivo*.

Table 3 – Effect of native and modified cyclodextrins in permeability of drugs though the skin.

Cyclodextrin	Effect(s)	Drug
α-CD	Increase the *in vitro* transdermal permeability.	Dehydroepiandrosterone[85]
β-CD	Increase the *in vitro* skin permeation.	Betamethasone[86], 4-Biphenylacetic acid[87], Dexamethasone[88], Flurbiprofen[89]
γ-CD	Increase the permeation in a skin-friendly way.	Beclomethasone dipropionate[90], Fludrocortisone acetate[91]
HPβCD	Increase the *in vitro* skin permeation.	Avobenzone[92], Curcumin[93], Dexamethasone[88], Estradiol[94], Ketoprofen[95], Oxybenzone[96 97]
Rameb	Improve the solubility and permeation; reduce the skin barrier function.	Bupranolol[98]

Cyclodextrin inclusion allows turning an oily ingredient into a solid, that is easier to manipulate and often more resistant to thermal degradation[99]. This way, plant essential oils

and liquid botanical extracts can be turned into powders and their volatility is strongly reduced. Besides these advantages, and given that the essential oil is a mixture of compounds, the cyclodextrin may perform a selective encapsulation of a few components. In the case of these being minor components in the pure essential oil, they may then become major components in the inclusion complex, which may lead to the emergence of interesting properties that in the oil were masked by the existence of in larger quantities of other components that do not manifest this property.

ICs are already commercially available in order to use them as ingredients in the cosmetic and pharmaceutical cosmetic (Table 4). Furthermore, it is known that there are several products already marketed with ICs as well as patents.

Table 4 – Cyclodextrin inclusion complexes used in commercially available cosmetic and medicines.

Inclusion Complex	Use	Ref
β-CD·citral	Used as a raw material in the perfume industry.	[100]
γ-CD·co-enzyme Q10	Recommended for use in cosmetic nutrition, as it is well absorbed by the skin in the presence of dipotassium glycyrrhizate.	[101,102]
2γ-CD·retinol	Recommended for personal and skin care, anti-aging and suncare products.	[103]
2γ-CD·vitamin E	Recommended for products within skin care, anti-aging, self-tanning, shaving formulations, coloured cosmetics and sun lotions.	[104]

Cyclodextrins are used as active ingredients for the entrapment of odours in the household deodorant spray "Febreze"[105], and in the laundry dryer sheets "Bounce"[106]. These two product lines have come to grow into the largest market applications for CDs in the cosmetics & toiletries branch. CDs are also present in sun lotions, protecting and stabilising the chemicals against precocious degradation from light and the aromatic compounds thus bringing a sustained release effect[107].

In addition to these products, there are still patents related to the use of cyclodextrins in cosmetics and in daily care products. A set of aqueous compositions based on cyclodextrins (and polymers as thickeners), which may or may not contain adjuvant adsorbent and antimicrobial agents, is claimed to reduce body odours[108] . In another patent, compositions based on cyclodextrins and surfactants are claimed to entrap odours by spraying on surfaces[109]. In a toothpaste formulation, β-CD is used in tandem with papain to achieve 'rapid removal of bad breath'[110].Transdermal films for the delivery of active

substances, having a composite of HPβCD, polyvinyl alcohol and chitosanes as the main ingredients, are already patented in China[111]. DIMEB and RAMEB are patented for promoting the penetration of vitamin A or its esthers in compositions intended for cosmetics or dermatology care, to 'regulate the cell metabolism of the skin, and/or preserve or restore a good physiological skin condition and/or improve the tone, firmness and elasticity of the skin, and/or delay the appearance of wrinkles or reduce their depth'[112].

In cosmetic formulations, in addition to the previously mentioned advantage, CDs modulate of the properties of creams and gels[82], and are good solubilizers for oily components since they form transparent aqueous solutions, whereas other dissolution agents typically cause cloudiness[99]. CDs also help formulate otherwise non-compatible components by inclusion and 'mask' of one of these components. CDs also help to reduce skin redness and irritation caused by active compounds, as celecoxib[113]and tretinoin[114], and making retinoids better tolerated[115].

Regarding aromas, CDs help mask unpleasant odours or flavours (of active ingredients such as omega-3 and omega-6 oils)[116,117], and they are excellent aroma stabilizers, helping maintain flavour in lipsticks, perfumes and room fresheners[118]. Inclusion complexes of aromatic compounds such as essential oils or their components present, besides the longer release of aroma, a much higher storage time and sheld-life. Hydrophilic gels containing HPβCD inclusion complexes with linalool, the main terpenic compound associated with lavender fragrance, and benzyl acetate, an aromatic compound associated with jasmine fragrance, were shown to retain the fragrance for over six months, while the same compositions having only linalool or benzyl acetate lost the fragrance after two months[99]. Sustained-release activity was demonstrated i*n vitro* for eugenol. The amount of this compound permeating excised skin 24h after application was roughly five times lower for HPβCD·eugenol than for pure eugenol[119]. Sustained release properties were also reported for β-CD·*trans*-anethole[120], HPβCD·ionone (sweet osmanthus flower fragrance)[121] and HPβCD·geraniol[122]. In parallel, compounds with a very high affinity towards the cyclodextrin cavity will dissociate at a very slow rate from the complex and thus these ICs will exhibit sustained release of the drug or active ingredient when applied on the skin.

The formation of IC lead to the protection of guest molecules as they can suffer several reactive reaction and this way the hydrolysis, oxidation, isomerisation, polymerisation, and enzymatic decomposition reactions are eliminated or minimised,

resulting in extended shelf-life for the products containing ICs[123]. This way, consumer safety is also increased by ensuring that all the components have their characteristics unaltered for a longer time. At the same time, the organoleptic characteristics do not change, which enables the continuous acceptance of the food or the cosmetic product. Considering all these advantages, the practical utility of inclusion complexes in food and cosmetics is self-evident.

1.2. *Cistus ladanifer*, a Mediterranean Plant

Cistus ladanifer, sometimes also referred to as *Cistus ladaniferus*[124], is a bushy plant of the genus *Cistus* and to subgenus *Leucocistus*. It is part of the *Cistaceae* family, a large family of Mediterranean plants comprising almost 200 species of shrubs[125]. *Cistus ladanifer* is the scientific name for the rockrose plant, known for its pentameric white flowers having a crimson spot in the base of the petals and a small yellow eye. The species of this family include fragrant and sweet smelling plants that are used in cosmetic and the perfume industry[126] and the leaves and the steams secrete an abundant exudate consisting of secondary metabolites, bioactive compounds, which are widely appreciated for the potential beneficial health effects.

Figure 2 – Cistus ladanifer flower[126].

1.2.1. Geographic Distribution

Cistus ladanifer grows all over the world, being particularly abundant in the Mediterranean region and the Iberian Peninsula[127]. This plant grows preferably on volcanic sandstone soil and in continental climate regions. In Portugal, it is possible to identify several areas where this species abounds. In a study carried out in 2008, 23 samples of *C. ladanifer* were collected in the Faro district, in the south of Portugal[128]. *C. ladanifer* also thrives near mining areas such as Caveira, Lousal and São Domingos, in the Alentejo region[129]. Note that albeit it is mainly found in the South of Portugal, it may also be spotted in some areas of the North of Portugal[130,131].

Figure 3 (adapted from online information[132]) depicts the distribution of *C. ladanifer* species and subspecies in the Mediterranean area[133]. This plant is distributed over the south

of Portugal, north Morocco, central and south Spain, south France, Corsica, north Algeria and Cyprus.

Figure 3 – Distribution of Cistus Species and Subspecies in the Mediterranean geographic area[133].

1.2.2. Chemical Composition of *Cistus ladanifer*

Depending on the solvents used and the type of extraction and conditions that are applied, it is possible to obtain extracts with different compositions: an extract rich in flavonoids is commonly obtained with water or/and other polar solvents such as ethanol and methanol, or even acetone; one rich in terpenes is normally obtained with non-polar solvents and hydrodistillation method. There are two types of terpenic extracts: the essential oil and the labdanum, which is thicker than the essential oil. However, it should be noted that any type of distillation approach can lead to the thermal degradation of the compounds or to formation of other compounds.

1.2.2.1. Cistus ladanifer Flavonoids

The exudate of *Cistus ladanifer* is composed fundamentally of compounds of phenolic origin[134,135]. The composition of the exudate varies the season, being richer in flavonoids during the summer and poorer in the winter[136]. Indeed, the summer season causes *C. ladanifer* to be exposed to various types of stress, such as high temperature and levels of UV, which leads to further development of secondary compounds.

Using water, ethanol, methanol or acetone, it is possible to obtain a flavonoid extract, which composition variates due to the solvent used[137]. The phenolic compounds synthesized by *C. ladanifer* are mainly aglycone flavonoids, namely apigenin, kaempferol 3-methyl

ether, apigenin 4'-methyl ether, apigenin 7-methyl ether and kaempferol 3,7-di-*O*-methyl ether, that overall can represent about 6% and 26% of the dry weight of the exudate[134].

The phenolic content of the extracts also differ according to the raw material used: flowers, fruits, stems or leaves[138]. Flowers were reported to have the highest phenolic content, optimally when using methanol, ethanol:water (50:50), methanol:water (50:50) or acetonitrile. In fruits, phenolic content was best extracted with water, ethanol or methanol:water (50:50). From stem, extraction was more rentable using ethanol or acetonitrile and, from leaves, extraction was best achieved using water, ethanol or methanol:water (50:50).

Besides the high content in flavonoids, *C. ladanifer* also has tocopherols, sugars and fatty acids in its composition[139]. Some terpenes may also be found in the flavonoid exudate as a result of the extraction method[135]. They represent 1% to 2% of dry weight of the exudate and they are diterpenes (oxocativic acid, 6-acetoxy-7-oxo-8-labden-15-oic acid and 7-oxo-8-labden-15-oic acid).

1.2.2.2. Labdanum

Labdanum is a rich sticky brown resin obtained from the cistus shrubs and it has a long history of use in herbal medicine and as a perfume ingredient. This resin is a natural fixer of essential oils and of the odoriferous essence obtained from the leaves[130]. The crude gum of labdanum is obtained by treating the plant with hot alkaline water to remove all waxes on the surface resinous matter and oily parts from the plant. Labdanum can be subdivided into three portions, depending on the extraction performed: the resinoid, that is obtained by steam distillation of the crude gum with ethanol[140], the concrete, that corresponds to the extract of the whole plant with hexane, and the absolute, obtained by alcohol extraction of the concrete[141].

The main components of resinoid labdanum are ledene, viridiflorol, cubeban-11-ol, borneol, copaborneol, bornyl acetate, *trans*-pinocarveol, eugenol, *allo*-aromadendrene and myrtenol[140]. These compounds are isoprene derivatives with fifteen to twenty carbons. In another study, the labdanolic acid, an important precursor of ambrox, a fixative perfume with amber odour widely used in the industry, was also identified[141]. Waxes can also be found in the resinoid and concrete.

1.2.2.3. Essential Oil of Cistus ladanifer

Cistus ladanifer essential oil is a mixture of volatile compounds with a strong odour. It is mainly constituted by secondary metabolites of the aromatic plant rockrose.

The essential oil is suitable for usage in perfumes, because it has a reminiscent fragrance of gray amber, which blends well with ambar and fern fregrances[126]. It can be obtained by a wide variety of methods. Extraction from fresh plant material by distillation for four hours yielded 0.3-0.4% ($^{m}/_{v}$) of essential oil[141], but the yield may be influenced by other factors, since a yield of 0.63% was reported for a three-hour extraction[142]. The essential oil may also be isolated by solvent extraction[130], supercritical carbon dioxide[143], steam distillation[144] and hydrodistillation[131], as listed in Table 5. A careful analysis of this tables allows noting the strong variation in the main components of *C. ladanifer* essential oil, which depend not only on the method of extraction, but also on geoclimatic factors such as soil composition, temperature and humidity, and on the stage of the vegetative cycle and the part of the plant used. Notably, the presence of α-pinene in almost all samples of essential oil is observed, as well as viridiflorol, verbanone and 2,2,6-trimethylcyclohexanone.

Table 5 – Summary of different extraction methods for obtaining essential oil of Cistus ladanifer, emphasising the main constituents that are obtained. Sample location and collection season are detailed, as well as the extraction condition. NC refers to the number of compounds of each sample.

Sample	Extraction	Analysis	NC	Main compounds	Ref
Corsica, Spain[£]	Hydrodistillation (24h)	GC ^{13}C-NMR	45	α-pinene, viridiflorol, ledol, bornyl acetate	[145]
Douro, Portugal (March)	Hydrodistillation (24h)	GC-MS, GC-sniffing, GC-MS-FID	14	Acetophenone, borneol, 2-phenylethanol, 2,2,6-trimethylcyclohexanone	[130]
Tafoughalt, Morocco (May)	Hydrodistillation (5h)	GC-MS	28	Camphene, borneol, terpin-4-ol, α-pinene, 2,2,6-trimethylcyclohexanone	[138]
Tanger, Morocco (August)	Hydrodistillation (4h)	GC-MS	68	α-pinene, camphene, bornyl acetate, ledol, viridiflorol 2,2,6-trimethylcyclohexanone	[141]
North Morocco (vegetative period)	Hydrodistillation (3h)	GC-FID GC-MS	58	1,8-cineole, viridiflorol, γ-terpineol	[146]
Central, Spain (October)	Hydrodistillation (3h)	GC-FID GC-MS	48	*trans*-pinocarveol, α-pinene viridiflorol, bornyl acetate, terpinen-4-ol, 2(10)-pinen-3-one, *p*-mentha-1,5-dien-8-ol,	[147]
Centre-interior Portugal (July-August)	Hydrodistillation (2h)	GC-FID GC-MS	25[*§]	Viridiflorol, globulol, α-pinene	[131]
North Portugal (July-August)	Hydrodistillation (2h)	GC-FID GC-MS	24 f 18 d[*§]	Viridiflorol, globulol, 15-nor-labdan-8-ol	[131]
Massif de l'Estérel, France (May)	Steam distillation (2h)	GC-MS	22	α-pinene, viridiflorol verbanone, 2,2,6-trimethylcyclohexanone	[144]
Ciudad Real, Spain (July)	Supercritical CO_2 (9 MPa, 40 ºC, d_p = 0.30 mm, F =0.7 kg/hr)	GC-FID	41	Camphor, α-pinene, borneol, camphene, γ-terpinene, thymol	[143]
Mirandela, Portugal (July)	Pentane simultaneous distillation-extraction (1h, 40 ºC)	HS-SPME GC-FID	4[#]	α-pinene, bornyl acetate, borneol, 2,2,6-trimethylcyclohexanone	[148]
São Domingos, & Corte do Pinto, Portugal (April)	Hexane extraction (30 minutes at 100 ºC, 1.38 MPa)	GC-MS	32[&]	Benzenepropanoic acid, viridiflorol, verbanone, globulol, α-pinene	[149]

Note:

[£] – without reference to the season, [*] – not all compounds identified,

[§] – comparative study between two different places and fresh (f) or dry (d) samples,

[#] – used ten target compounds and identified four of them in the sample,

[&] – same compounds identified but in different percentage.

1.2.3. Compounds of *Cistus ladanifer* Essential Oil with Known Biological Activity

C. ladanifer essential oil is used in traditional medicine for the treatment of various skin diseases, as an anti-diarrheic and as anti-inflammatory agent[139]. The medicinal properties are related to the presence of various active components, of which three have already been studied isolatedly.

1.2.3.1. α-Pinene

Alpha-pinene (2,6,6-trimethylbicyclo(3.1.1)-2-hept-2-ene) (Figure 4) is an organic terpene compound with anti-inflammatory properties. It is found in the oils of a variety of plants (Table 6), with highlight to coniferous trees of which the most well-known is *Pinus silvestris*[150,151]. It has been approved as a food additive with GRAS status[152].

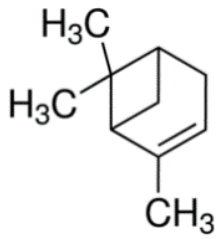

Figure 4 – The chemical structure of α-pinene[153].

Table 6 – Percentage of α-pinene in different plants essential oils.

Plant	α-pinene (%)	Ref
Cannabis sativa	23 ± 17	[154]
Echinophora cinerea	26.54 ± 0.21	[155]
Elaeoselinum asclepium	43.9	[156]
Eucalyptus camaldulensis	15.6	[157]
Ferula tunetana	39.8	[158]
Melaleuca leucadendron	12.22	[159]
Pinus sylvestris	16.1	[151]
Pistacia vera	77.5	[160]
Rosmarinus officinalis	19.43 – 21.3	[161,162]

The anti-inflammatory activity of α-pinene was evaluated under two scenarios of induced inflammation. In carrageenan-induced inflammation in mice, α-pinene extract (77.5%) from pistachio was administered in various dosages with those of 250 and 500

mg/kg being found to reduce inflammation and appearance of gastric lesion[160]. In a cerulein-induced acute pancreatitis in mice, treatment with α-pinene reduced the pancreatitic tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, *in vivo*, and inhibited cerulein-induced cell death and cytokine production, *in vitro*[163].

A study with mice has indicated a possible application of α-pinene as an anxiolytic substance. Mice were submitted to the elevated plus maze test for 10 min, after having inhaled α-pinene for 90 min/day for 1 day, 3 days, or 5 days[164]. The concentrations of α-pinene in the brain and liver were quantify and there was significant anxiolytic-like activity, which remained constant for the 5 days after inhalation. Thus, α-pinene appears to have biological properties of interest.

1.2.3.2. Camphene

Camphene (IUPAC: 2,2-dimethyl-3-methylidenebicycloheptane) (Figure 5) is a bicyclic monoterpene, i.e., an organic compound formed by two fused cycloheptane rings (alipahtic six-member rings formed only of carbon atoms)[165], occurring in high quantities the essential oils of *Artemisia terrae-albae*[166] and *Rosmarinus officinalis*[162 161] and smaller or trace amounts in the essential oils of *Elaeoselinum asclepium*[156], *Ferula tunetana*[158], *Hertia cheirifolia*[167], *Lavandula officinalis*[168], Cardomom[169], *Cannabis sativa*[170]. It can be used as flavouring agent, having a woody, camphoreous note and there is no safety concern at current levels of intake, having an acceptable ADI (concentration not mentioned)[171].

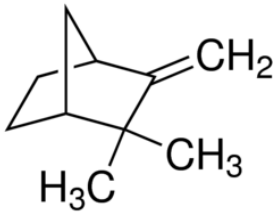

Figure 5 – The chemical structure of camphene[172].

Camphene is currently still under evaluation regarding the medicinal applications, but available data already demonstrates a variety of activities. It was shown to have a cytoprotective antioxidant effect on *t*-butylhydroperoxide-stressed alveolar macrophages by decreasing lipid peroxidation and the release of NO and reactive oxygen species, while the production of superoxide dismutase and glutathione were increased[173]. This study provides

evidence for the traditional medicine claims, 'the aroma is part of the healing', since it demonstrated that in fact this particularly odoriferous component of *C. ladanifer* is beneficial to the organism. Camphene may also be used as cholesterol-reducing agent, since it was demonstrated to inhibit HMG-CoA reductase, the cellular enzyme required for cholesterol biosynthesis[174].

In topical formulations, camphene overall well tolerated at doses up to 4%, with no irritation observed after a 48-hour closed-patch test[175]. It also displayed no cytotoxic effects on HeLa cells in monolayer culture *in vitro* test[176].

1.2.3.3. p-Cymene

p-Cymene (IUPAC: 1-methyl-4-(propan-2-yl)benzene) (Figure 6) is an alkylbenzene related to a monoterpene found in gymnospermic and angiospermic plants[177,178].

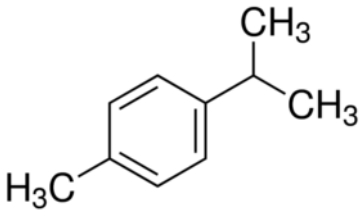

Figure 6 – The chemical structure of p-cymene[179].

This compound is well absorbed through the skin. In studies with ^{14}C-labelled *p*-cymene, the penetration observed was 254 ug/sq cm in 60 minutes[180]. This maximization test was carried out on 25 volunteers, *p*-cymene was tested at 4% concentration in petrolatum and produced no sensitization reactions during 48 hours.

In mice with LPS-induced acute lung injury, *p*-cymene reduced significantly the oedema, congestion and inflammation by lowering pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), interfering with myeloperoxidase activity, and blocking several inflammatory proteins such as mitogen-activated kinase (MAPK)[178].

1.2.4. Biological Activities of *Cistus ladanifer*

The *C. ladanifer* is probably the most well-characterised of all *Cistus* species. For other *Cistus* genus, it is established that the flavonoid extract have antioxidant activity and protective effect on DNA cleavage[181]. A study performed in 1993 hypothesised that the flavonoid extract from *C. ladanifer* could protect against the damaging effects of near-UV-visible solar radiation[136], based on the ability of these flavonoids to absorb light at this wavelength interval. However, there is no current reference regarding developments of the application of *C. ladanifer* as a UV filter.

1.2.4.1. Antioxidant Activity

The flavonoid extract of *Cistus ladanifer* was extensively study in relation to the antioxidant activity, by different tests, such as Folin-Ciocalteu, TEAC, FRAP, ORAC and TBARS[182]. On the other hand, as terpenic compounds have low antioxidant activity, the essential oil is not widely study. There is only one reference to a DPPH study for *Cistus ladanifer* essential oil, which is referenced in Table 7, as well as for three phenolic extracts.

Table 7 – EC_{50} values for DPPH assay for extracts of Cistus ladanifer.

Extract	EC_{50} /mg/mL	Ref
Hydroethanol[*] (95:5, v:v)	0.00785[§]	137
Hydroacetone[*] (60:40, v:v)	0.03951[§]	
Methanol[*] 100%	0.13 ± 0.22	142
Essential oil extract[#]	36.28 ± 0.36	

Note:
[*] – phenolic extract, [§] – values do not have a confidence range,
[#] – extract obtained by hydrodistillation.

Note how the EC_{50} values of the phenolic extracts differ according to the co-solvent used in the extraction. The EC_{50} is about five times higher for the acetone extract and about sixteen times higher for the methanol extract. This means that the phenolic extract of *Cistus ladanifer* using ethanol is the most effective in scavenging the free radical DPPH. On the other hand, the value for the essential oil test is about 280 times higher than that of the methanol extract, which means that a concentration of about 280 times more is required for the oil to have the same effect as the methanol extract; in other words, the methanol extract is more effective than the essential oil in the same concentrations. For the others extracts,

the difference between the essential oil extract and the ethanol and the acetone extracts would be even higher.

1.2.4.2. Antimicrobial Activity

Cistus ladanifer essential oil has antimicrobial activity on a few bacterial and fungal strains[141,183], as summarized in Table 8. It should be noted that the data is obtained from two different studies, one evaluating the MIC[141] and the other one measuring the inhibition diameter[183].

Table 8 – Antimicrobial activities of Cistus ladanifer essential oil on bacteria and fungi strains.

	Tested Micro-organism	**Conclusion[£]**
Gram Negative	*Citrobacter* sp.[183], *Pseudomonas aeruginosa*[183], *Salmonella typhi*[183]	0
	Enterobacter cloacea[183], *Escherichia coli*[141,183], *Klebsiella pneumoneae*[183], *Proteus mirabilis*[183]	+
Gram Positive	*Bacilus megaterium*[141]	+
	Listeria monocytogenes[183], *Staphylococcus aureus*[183]	++
Fungi[141]	*Aspergillus niger, Botrytis cinerea, Mucor recemosus, Verticillium albo-atrum*	+

Note:
[£] – the assays performed on the studies where different.

It was considered (+) for the two studies in which antimicrobial action of the essential oil was verified. In the case where this action was extensive, it was denominated with (++) in which the diameter of inhibition was superior to 10 mm[183].

The essential oil manifested antimicrobial activity against Gram-negative and Gram-positive bacteria and fungi strains. Only three Gram-negative bacteria strains did not respond to the activity of the essential oil, while Gram-positive bacteria *Listeria monocytogenes* and *Staphylococcus aureus* displayed an excellent response to the essential oil as they exhibited the largest zones of inhibition. Taking into account the properties described, it would be interesting to apply *C. ladanifer* essential oil in cosmetic products.

1.2.4.3. Cistus ladanifer Other Biological Activities

Cistus ladanifer may also have herbicidal activity. An analysis of the areas cultivated with this plant showed that flavonoid extracts secreted by its leaves inhibited and delayed the germination of herbaceous species. So, *Cistus ladanifer* essential oil has been tested against weed germination. Inhibition was achieved against germination of *Amaranthus hybridus, Conyza canadensis* and *Parietaria judaica*, at 0.125 μL/mL, and the germination of *Portulaca oleracea* was controlled, using 1 μL/mL, while no effect was observed against *Chenopodium album*[147]. In this way, *Cistus ladanifer* essential oil can be used as natural and selective herbicide, although the herbicidal concentrations have yet to be established.

1.2.5. Encapsulation of *Cistus ladanifer* Essential Oil

For centuries, *Cistus ladanifer* essential oil has a tradition of use in skin ointments, being described in folklore as one of the twelve oils mentioned in the ancient scriptures[184] and claimed to possess regenerative and immuno-regulating abilities. Although these properties are still to be demonstrated clinically, *Cistus ladanifer* is already found in the market in an eye-contour formulation, in a blend with other essential oils[185].

As the essential oil has the characteristic fragrance of the plant, the inclusion complex may also have this fragrance. The application of the inclusion complex in certain products may lead to the consideration that the product also has pharmacological activity, as it may occur in creams. The desirable application for this inclusion complex would be in acne creams. Acne is caused by the Gram-positive bacteria *Propionibacteria acnes*, so this cream would have a cosmetic and pharmacological application[186]. A skin application for *Cistus ladanifer* is already patented[187]. The patent claim improved topical analgesic action for a combination of *Cistus ladanifer* and *Cupressus sempervirens* terpene-containing extract with *Acellella oleracea*. Note also that no adverse reaction are reported for the oil. Only one case of contact dermatitis is reported until now, but it happened for contact with the fresh plant[188]. The symptomatology may thus have been caused by some particular compound or by the structures of the plant leaves.

1.3. Ginger Rhizome, an Indian Spice

Zingiber officinale, commonly known as ginger, is an Indian native perennial plant with thick tuberous rhizomes and stems rising almost one meter from the ground. Its rhizome is widely used in food and traditional oriental medicine[189]. Dried and grounded to a powder, it is used as a spice of flowery-spicy taste and aroma. As a medicinal plant, for example, it is used to facilitate digestion, stimulate the circulatory system and help with morning sickness and colds.

Figure 7 – Ginger rhizome and powder[190].

1.3.1. Chemical Composition of Ginger Rhizome

From the ginger rhizome it is possible to obtain several extracts with varied compositions. Using *n*-hexane and methanol, it is possible to obtain an extract with 6- and 10-dehydrogingerdione and 6- and 10-gingerdione[191], whereas extraction with acetone gets 6-gingerol[192]. One of the compounds with the most interest is 6-gingerol, which is widely distributed on the plant. Thus, one gram of fresh ginger rhizome and one gram of ginger root were grown in different laboratory conditions, as well as stems[193], and, in all the extracts, 6-gingerol was detected, but the one that allows a better extraction is the fresh rhizome of ginger.

The pungent compounds comprise 6-, 8- and 10-gingerols and 6-shogaol. 6-gingerol is found in larger amounts and it thus contributes more significantly to this characteristic[194]. The extraction of the various components of the rhizome can be done with the help of different solvents. 6-gingerol is more soluble in organic solvents, whereby the use of acetone becomes more advantageous for a better yield of 6-gingerol. Using acetone and methanol,

7.39% ($^{w}/_{w}$ of fresh ginger) and 5.69% are obtained, respectively[195]. In a 1:1 methanol:water mixture, 5.15% is obtained, while using water the yield is lowered to 4.58%.

Variation of temperatures and pressures as well as the flow of carbon dioxide allows an increase on extraction yield and the content in 6-gingerol[196]. The optimum condition for ginger oil yield was achieved at extraction pressure of 15 MPa, temperature of 35 ºC and CO_2 flowrate of 10 g/min, while the optimum condition to obtained 6-gingerol was at extraction pressure of 10 MPa, temperature of 35 ºC and CO_2 flow rate of 10 g/min.

An extraction was performed starting from fresh ginger and purchased oleoresin and 6-gingerol and 6-shogaol were isolated[197]. Comparing the most common pungent compounds, 6-shogaol is found to be higher in oleoresin extract, as well as 8- and 10-shogaol. These compounds correspond to 6-, 8- and 10-gingerol by conversion, forming a C=C double bond with loss of one molecule of water. Considering that the concentration of shogaols increases in oleoresin, it is possible to assume that the increase of shogaol presents as a change in ginger extract, thus being a method of identifying degradation.

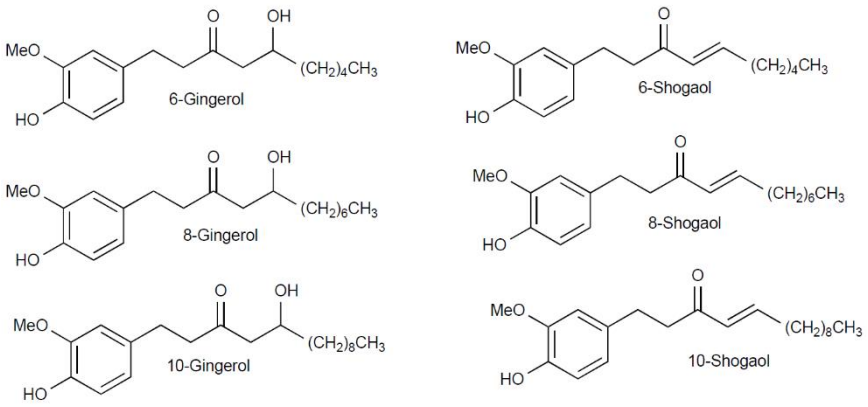

Figure 8 – Structure of 6-, 8- and 10-gingerol (left), and 6-, 8- and 10-shogaol (right).

HPLC-UV at 230 nm and 280 nm was performed, as the variation in the absorbance allowed to identify the loss of data in the reading at 230 nm[197]. The variation in the absorbance between 230 nm and 500 nm for 6-gingerol and 6-shogaol, allows identification of the shogaols family, as consequence of the appearance of the unsaturated bond. Thus, reading at 230 nm on HPLC-UV allows identification of the shogaols family.

1.3.2. Biological Activities of Ginger Rhizome

Due to its abundance, low cost and safety in consumption, ginger has been the subject of intense scientific research over the past two decades. With scientific developments, ginger and specifically its compounds have shown anticancer, antibacterial, antifungal, hypoglycemic and antiatherosclerotic features.

The consumption of ginger by those who are not accustomed to it may cause some temporary malaise, due to the spicy flavour, but there is no known acute toxicity in the doses of regular consumption, both in food and for medicinal purposes. On the other hand, very high doses (from 6 g) can lead to gastric irritation and loss of protective gastric mucosa. When administered, ginger was well tolerated up to a dose of 2.5 g/kg, with no mortality or side effects – except for diarrhea in two animals[198]. At doses of 3.0 g/kg and 3.5 g/kg, deaths (20 – 30%) and other secondary symptoms such as gastrointestinal spasm, hypothermia, diarrhea and anorexia occurred (30 – 60%).

One case of contact dermatitis was reported related to ginger[199]. Ginger is on the FDA's GRAS list and its essential oils, oleoresins (solvent-free) and natural extractives (including distillates) are generally recognized as safe for their intended use, within the meaning of section 409 of the act[200]. 6-Gingerol is not considered cytotoxic, as it showed no effect on the viability of 3T3-L1 pre-adipocyte cell cytotoxic when tested in a concentration range of 1 ng/mL at 100 μg/mL for 24 h[201].

1.3.2.1. Gastrointestinal and Hepatic Activity

Ginger, and the various extracts obtainable therefrom, have activity on the gastrointestinal tract. In the mouth, ginger stimulates the production of saliva, which enables swallowing. The digestion mechanism in the remaining gastrointestinal tract has been understood using animal systems[202]. It stimulates bile acid production and secretion and the activity of digestive enzymes of pancreas-lipase, amylase and proteases – namely, trypsin, chymotrypsin and carboxy peptidase[203]. In the small intestine, digestive enzymes also benefit from a diet with ginger[204]. Table 9 shows the data collected from the three tests mentioned, referring to the digestive enzymes activity *in vivo* studies in rats.

Table 9 – Summary table of results for pancreatic and intestinal enzyme activity in vivo studies with rats.

Enzymes		Effect[204]	Effect[203]		Effect[205]
		Dietary	*Dietary*	*Singular*	*Dietary*
Pancreatic	**Lipase**		+*	–*	+*
	Amylase		+*	–*	+*
	Trypsin		+*	–	+*
	Chymotrypsin		+*	–	+*
Intestinal	**Lipase**	+*		+*	+*
	Amylase	~		+*	+*
	Alkaline phosphatase	+		+	
	Acid phosphatase	+*		–	
	Sucrase	+		+*	–
	Lactase	–		+	+*
	Maltase	+*		–	+*

Note:

+ – value superior to control, **–** – value inferior to control, **~** – value equal to control,

* – value statistically different from control.

The continuous consumption of ginger for eight weeks led to the increase of the activity of the four pancreatic enzymes mentioned in the table[203,205]. On the other hand, the unique consumption of ginger leads to the decrease of the activity for all the pancreatic enzymes referred[203]. Thus, continued supplementation with ginger leads to an increase in pancreatic enzymes manifested by facilitated digestion and decreased intestinal transit time.

There is an increase in the activity of intestinal lipase, whether it was continuous supplementation or daily intake. The intestinal amylase activity increased with ginger supplementation[205], but this increase was not revealed in the previous study[204]. As the experimental supplementation conditions were not the same, there is no comparison of data between studies.

Phosphatase activity was only statistically significant for acid phosphatase even though alkaline phosphatase activity also increased[204]. In the case of disaccharide enzymes, there is a variety of results for sucrase and lactase, leading to the question of whether the feed previously performed will or not also influence this enzymes activity[204,205]. Intestinal maltose was the only disaccharide enzyme that activity increased with ginger dietary supplementation. The intestinal peptidic enzymes undergo changes with a diet supplemented with ginger. Consumption of ginger leads to increased activity of glycyl-glycine dipeptidase, leucine amino peptidase and γ-glutamyl transpeptidase[206].

Prakash and Srinivasan analysed the effects of ginger in the gastrointestinal tract in relation to the protective effect on the mucosa and the change in permeability. After ginger supplementation (0.05%) for eight weeks, there was a change in fluidity and permeability of the intestinal membrane, associated with increased length and perimeter of microvilli, resulting in an increase in the absorptive surface of the small intestine of rats[206]. With this same ginger supplementation for eight weeks, in an *in vitro* test, it was found that it lead to a greater absorption of iron, zinc, calcium and β-carotene[207]. Thus, dietary ginger exerts a beneficial influence on the gastrointestinal tract both in the enlargement of the contact surface for absorption, as well as the increase of the absorption rate, facilitating the intake of micronutrients.

An ethanolic ginger extract (500 mg/kg) was shown to protect against ulcer formation by action of ethanol (80%), HCl (0.6 M), NaOH (0.2 M), NaCl (25%), indomethacin (30 mg/kg body weight) and aspirin (200 mg/kg body weight) when administered to rats 30 minutes prior to induction of the gastric lesion; no protective effect was observed on reserpine-induced gastric mucosal injury[208]. However, the composition of this extract was not ascertained. In another study, 6-gingerol (0.09%), was shown to reduce whatever-induced stomach injuries by 34.8% when administered at a dose of 50 mg/kg and by 54.5% for a dose of 100 mg/kg[192].

In addition to the lesions induced with necrotic agents, these can also be induced by nonsteroidal anti-inflammatory drugs, such as indomethacin, aspirin and reserpine[208]. Supplementation with 500 mg/kg ethanolic extract of ginger (unknown composition) prior to administration of the inducing drug led to a decrease in gastric mucosal injury in the case of indomethacin and aspirin-induced injuries, but had no effect in reserpine-induced injury.

The consumption of ginger is typically associated with an antiemetic activity[209], even though there is no corroborating data. Furthermore, treatments with ginger did not reduce the incidence of nausea and vomiting in post-operative situations[210,211].

1.3.2.2. Antimicrobial Activity: Antibacterial and Antifungal

Ginger and its extracts are given credit for antimicrobial activity, which may be important if ginger is used as a natural additive. Analysis of two literature studies claiming this has, however, shown the extracts were obtained by subcritical extraction with water[212] and ethanol[198] and their composition was not assessed. Given the low solubility of gingerols

in these solvents, the extracts most likely do not contain these actives. Furthermore, the ginger extract has higher MICs when compared to the control, so the amounts needed to have an effective inhibition would be very high (between 80 and 160 times more)[212]. Thereby it is not possible to corroborate the antimicrobial activity of ginger and its components.

1.3.2.3. Anti-inflammatory Activity

Inflammatory activity results from the action of the enzyme cyclooxygenase (COX) or the synthesis of inflammatory prostaglandins. The use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces inflammation, by enzymatic inhibition or synthesis.

Various assays are carried out aiming to inhibit the enzyme, to lead to a decrease in the expression of a particular inflammatory compound or to observe the physical changes carried out by an inflammation. The enzymes and substrates associated with inflammatory activity – PGE_2, PG synthetase and 5-LOX –, both *in vitro* and *in vivo*, and the development of edema were studied. Table 10 summarizes the information regarding the anti-inflammatory activity in ginger powder rhizome and gingerols, considering the various types of tests performed and the conclusions achieved.

Table 10 – Summary table of anti-inflammatory activity of ginger powder rhizome and gingerols.

	Activity	**Dose → Effect**	**Ref**
Ginger	Reduction of PGE_2 serum *in vivo*	Orally 50 mg/kg Intraperitoneally 500 mg/kg	213
	Carrageenan-induced edema reduction	50 mg/kg → 22 % 100 mg/kg → 33%	198
Gingerols	PG synthetase inhibition *in vitro*	EC_{50} = 4.6 μM 6-gingerol EC_{50} = 5.0 μM 8-gingerol EC_{50} = 2.5 μM 10-gingerol	214
	Reduction of PGE_2 production *in vitro*	58% 6-gingerol 66% 8-gingerol 73% 10-gingerol	215
	5-LOX inhibition *in vitro*	EC_{50} = 30 μM 6-gingerol EC_{50} = 0.36 μM 8-gingerol EC_{50} = 0.053 μM 10-gingerol	214

It is necessary to mention that a bibliographical research about carragenan-induced edema with 6-gingerol done[216], but after some analyze, it was found that there was no compliance between the concentrations of 6-gingerol referenced in the text with the

information in the chart, added to an incorrect interpretation of a significant decrease in edema. However, since the carrageenan-induced edema test is a test of anti-inflammatory activity and a validated one, it was considered important to make a brief reference[217,218].

Ginger has also been claimed to have anti-thrombotic features, since TXB_2 levels were significantly decrease from oral administration of 500 mg/kg in rats[213]. Additional, antipyretic activity is considered as supplementation of rats for four hours with 100 mg/kg ginger ethanolic extract was found to reduce body temperature by 38% while the same dose of acetylsalicylic acid was effective in 44%[198]. Thus, the administration of ginger may act as an anti-inflammatory and it is a clinically important development since its administration does not cause side effects nor complications with non-steroidal anti-inflammatory drugs.

1.3.2.4. Antioxidant Activity

In order to analyze the effects of ginger as a dietary supplement for malathion-induced lipid peroxidation occurs, 1% (w/w) of ginger powder was added to standard laboratory diet and a feed containing malathion (20 mg of malathion in 1 kg of standard feed) was also prepared[219]. It was tested lipid peroxidation by TBARS, superoxide dismutase (SOD) and catalase (CAT) activity and the glutathione related enzymes: total glutathione (GSH), glutathione reductase (GR), by analysis the oxidation of NADPH to NADP, glutathione peroxidase (GPx) and serum glutathione S-transferase (GST) in blood samples. The results are reported in Table 11.

Table 11 – Summary table of tests results carried out in antioxidant activity enzymes.

	Ginger	**Malathion**	**Malathion+Ginger**
Lipid peroxidation	– [a]	+ [ab]	– [c]
SOD activity	–	+ [ab]	+ [abc*]
CAT activity	– [a]	+ [ab]	~ [bc]
GSH content	+ [a]	– [ab]	+ [c]
GR activity	–	+ [ab]	~ [c]
GPx activity	–	+ [ab]	– [c]
GST activity	–	+ [ab]	+ [bc]

Note:

+ – value superior to control, **–** – value inferior to control, **~** – value equal to control,

[a] – different from control, [b] – different from ginger, [c] – different from malathion, [*] – inferior to malathion.

The activity of malathion seems to be contraire to ginger and the malathion-diet supplemented with ginger. The results of this study suggest that ginger exerted an antioxidant effect, reducing lipid peroxidation, increasing GSH content and maintaining normal levels of antioxidant enzymes. High levels of antioxidant enzymes can be correlated with increased lipid peroxidation in the case of malathion feeding. Ginger leads to an indirect increase in GSH levels, increasing the amount of GTS. Analysis of the various glutathione-related enzymes shows that GPx activity is correlated with GR activity, which provides substrate to the GPx enzyme, leading to an increase in the activity of the other enzymes as well.

Several *in vitro* assays evidence the antioxidant activity of gingerols and 6-shogaol, as it can be noticed in Table 12.

Table 12 – Summary table of antioxidant activity of gingerols and 6-shogaol.

Assay		**Values**	**Ref**
Free Radical Scavenging		EC_{50} = 4.05 μM 6-gingerol	[215]
		EC_{50} = 2.50 μM 8-gingerol	
		EC_{50} = 1.68 μM 10-gingerol	
		EC_{50} = 0.85 μM 6-shogaol	
Hydroxyl Radicals Produced by Fe(II)-H_2O_2		EC_{50} = 4.61 μM 6-gingerol	[215]
		EC_{50} = 1.97 μM 8-gingerol	
		EC_{50} = 1.35 μM 10-gingerol	
		EC_{50} = 0.71 μM 6-shogaol	
DPPH	*steady state at 180 min*	20 μM 6-gingerol	[220]
		6-shogaol > 10-gingerol > 8-gingerol > 6-gingerol	[215]
	on DMSO 30 min at 37 °C	EC_{50} = 26.3 ± 1.42 μM 6-gingerol	
		EC_{50} = 8.05 ± 1.02 μM 6-shogaol	
β-Carotene Bleaching		BHT=VE > 6-gingerol > MD-R=SFE-O > MD-EO	[221]

Note:
BHT – butylated hydroxytoluene; VE – vitamin E; MD-R – molecular distillation residue;
SFE-O – supercritical fluid extracted oleoresin; MD-EO – molecular distillation of the essential oil.

It is found that there is a vast analysis of the antioxidant activity of ginger and gingerols. As there is some variation between values related to the same assay, it can be seen that the specification of the composition and the solvent used influence the data. Therefore, the comparison with the laboratory data should take this into account. It is also needed to mention that the presence of the unsaturated bond in 6-shogaol may possibly lead to a higher antioxidant power and the length of the carbon chain also has an influence on the activity.

1.3.2.5. Hypoglycemic Properties

Diabetes mellitus is one of the major metabolic disorders and a growing affliction in modern societies to which consumption of ginger may bring helpful health benefits. Ginger ethanolic extract with unknown composition showed hypoglycemic potential in rabbits. Doses of 100 and 300 mg/kg reduced glycemia by 46.1% and 51.4%, respectively, two hours following administration and the levels being maintained for another two hours[198].

In rats, results are also promising. A study involving diabetic hypercholesterolemic rats shown that supplementation with 3 g ginger for 30 days led to a significant reduction of blood glucose levels[222]. Another study with glucose intolerant diabetic rats with daily supplementation of 100 mg/kg of 6-gingerol for 12 days lead to a significant lowering of glucose levels after 90 minutes of glucose ingestion, which did not occur in untreated subjects[201]. Liver and renal (CAT, GPx and SOD) enzymes were also analyzed as well as malondialdehyde (MDA) concentration, by TBARS assay – data in Table 13.

Table 13 – Impact of 6-gingerol (100 mg/kg) on hepactic and renal antioxidant enzymes (U/mg protein) and MDA concentration (mmol/L) in rats[201].

	Liver			**Kidney**		
	db/+	db/db	6-gingerol	db/+	db/db	6-gingerol
CAT	10.5 ± 1.80	6.20 ± 2.50 $^{\#\#}$	8.98 ± 2.40***	8.5 ± 2.10	5.70 ± 2.50 $^{\#\#}$	7.50 ± 2.40*
GPx	65.4 ± 3.50	48.5 ± 2.90 $^{\#\#}$	60.1 ± 2.70*	52.4 ± 3.50	38.5 ± 3.10 $^{\#\#}$	47.0 ± 3.70**
SOD	20.5 ± 2.50	14.2 ± 1.90 $^{\#\#}$	18.2 ± 2.30**	23.0 ± 2.90	18.2 ± 2.30 $^{\#}$	21.3 ± 1.90*
MDA	6.50 ±1.40	10.5 ± 1.50 $^{\#\#}$	7.20 ± 1.80**	8.22 ± 1.80	12.6 ± 1.20 $^{\#\#}$	9.60 ± 1.50*

Note:
db/+ – non diabetic; db/db – diabetic;
in relation to db/+ (non diabetic) rats: $^{\#}$ – $p < 0.05$, $^{\#\#}$ – $p < 0.01$,
in relation to db/db (diabetic) rats: * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$.

In the liver of rats with diabetes the supplementation with 6-gingerol increased CAT (44.8%), GPx (23.9%) and SOD (28.1%) activity. An increased of kidney enzymes activity was also observed: CAT (24.0%), GPx (22.1%) and SOD (17.0%). It was observed that 6-gingerol treatment conferred a significant reduction in lipid peroxidation in the liver (31.4%) and in the kidney (23.8%), when compared to diabetic rats. In diabetic rats, 6-gingerol reduced the reactive oxygen spices content from 262.8% to 64.0%, after 12 days of treatment. The major outcome of this study is demonstrating that the supplementation with 6-gingerol allows the reduction of the activity of antioxidant enzymes, without causing

adverse effects to the individual. Supplementation with 6-gingerol also significantly reduced body weight from 50.4 ± 8.9 g on day 0 to 43.5 ± 7.8 g on day 12 in diabetic rats. 6-gingerol manifests ability to lower blood sugar, reduce body weight and change the production levels and plasma insulin concentrations.

Ginger can inhibiting advanced glycation end products (AGEs) formation *in vitro* and *in vivo*, through its antiglycatory potential[223]. Blood glucose and insulin levels were evaluated to understand whether ginger improved the onset and progression of cataract, a pathological condition associated with the development of AGEs. With ginger supplementation, blood glucose levels declined to a low to moderate level, depending on the dose given to diabetic rats, while insulin levels were not affected. Thus, the delay in the progression of cataracts in diabetic rats due to ginger feeding may be attributed to their potential to modulate pathways.

1.3.2.6. Cholesterol-lowering Properties

Ginger is also being evaluated as a cholesterol regulating agent. There is a great concern with high levels of cholesterol, in particular concerning the role of low density lipoprotein cholesterol (LDL-C) it pathogenesis of atherosclerosis.

The antihypercholesterolemic effects of ginger at various doses (Table 14) was evaluated in rats fed for previous 8 weeks with a high cholesterol feeding diet containing 1% cholesterol powder + 0.2% cholic acid + 10% fat[224].

Table 14 – Effect of oral administration of aqueous ginger infusion (100, 200 and 400 mg/kg) in serum triglycerides, total, LDL and HDL cholesterol[224].

	Normal control	**Hypercholesterolaemic control**	**Ginger 100 mg/kg**	**Ginger 200 mg/kg**	**Ginger 400 mg/kg**
Triglycerides	10.32% ↓	2.99% ↑	42.53% ↓	84.28% ↓	90.49% ↓
Total cholesterol	0.45% ↓	6.82% ↑	70.85% ↓	69.41% ↓	77.96% ↓
LDL cholesterol	2.27% ↓	2.81% ↑	96.48% ↓	97.86% ↓	98.56% ↓
HDL cholesterol	2.33% ↑	54.92% ↓	29.83% ↑	39.39% ↑	29.86% ↑

Ginger infusion supplementation led to a reduction in serum triglyceride levels, total cholesterol and LDL cholesterol at all the tested doses, and in a dose-dependent manner for triglycerides and total cholesterol. On the other hand, the increase in ginger infusion supplementation did not lead to a more pronounced reduction of lipid LDL cholesterol.

Serum HLD cholesterol levels increased, having most notable increase for supplementation at 200 mg/kg. In parallel, a hypercholesterolaemic diet without supplementation led to an increase in triglycerides, total cholesterol and serum LDL cholesterol, and a decrease in HDL cholesterol. Ginger infusion at any of the concentrations led to a reduction in serum lipid levels.

Seric cholesterol and triglycerides may also be associated with complication of diabetes. The effect of supplementation with 6-gingerol at 100 mg/kg for 12 days in diabetic rats showed a significant reduction in plasma triglyceride levels (41.1%), total cholesterol (31.2%), LDL (27.9%) and free fatty acids (24.4%)[201]. Comparing with the values presented in Table 14, where 100 mg/kg of an aqueous infusion of ginger was given, only the reduction of triglycerides was similar. It can thus be assumed that other components of the ginger rhizome and the time of supplementation influence the reduction of lipidic serum profile.

Since high blood cholesterol is a risk factor for cardiovascular disorders, the hypocholesterolemic property of ginger should be indicative of its cardioprotective function. This was demonstrated using ginger ethanolic extract, that was able to prevent experimental isoproterenol-induced myocardial infarction[225]. This activity is related to the major active components of ginger, which comprise c.a. 51.7% of the extract composition (6-gingerol 249.15 μg/mL, 8-gingerol 38.08 μg/mL, 10-gingerol 67.85 μg/mL, 6-shogaol 79.41 μg/mL, 8-shogaol 12.24 μg/mL and 10-shogaol 34.81 μg/mL). Furthermore, pretreatment with ginger ethanolic extract 400 mg/kg for four weeks significantly decreased cardiac markers in rats – cardiac troponin I (cTnI), creatine kinase MB isoenzyme (CK-MB), lactate dehydrogenase (LDH), alanine transaminase (ALT) and aspartate transaminase (AST)[225]. Histopathologically, the control rats' myocardium showed a normal morphological architecture with no evidence of necrosis, whereas in isoproterenol induced rats showed signs of necrosis, with infiltration of numerous inflammatory cells, as well as the separation of muscle fibers. Pretreatment with ginger extract at various concentrations showed decreased myocardial damage at 100 mg/kg and 200 mg/kg, while the myocardial structure with the pretreatment 400 mg/kg was almost comparable with the control group.

1.3.2.7. Cancer Preventive Properties

The cancer-preventive activity of 6-gingerol is demonstrated by several *in vitro* and *in vivo* studies. Early reports used ginger and extracts of unknown composition, but, more recently, studies focused on the active compound 6-gingerol. *In vitro*, it is known that 6-gingerol inhibited the viability of human HL-60 (promyelocytic leukemia) cells[226] as well as the EFG-induced cell transformation and AP-1 activation in JB6 cells[227], and nitric oxide synthase (NOS) expression in LPS-treated cell lines[228], angiogenesis of human endothelial cells[229] and AP-1 transcriptional complex in human skin keratinocytes cell lines[230].

In addition to the *in vitro* studies, *in vivo* studies were also conducted to evaluate ginger rhizome preventive character against cancer as well as other components *per se*. In *in vivo* studies in rats, 6-gingerol inhibited cell proliferation and induced apoptosis in colon cancer cells, not affecting normal cells (ERK1/2/JNK/AP-1 pathway inhibition)[231]. In skin related cancers, 6-gingerol supressed progression mouse skin carcinogenesis[232] and inhibited TPA skin tumour promotion and epidermal ornithine decarboxylase activity in ICR mice[226]. It exhibited anti-tumour-promoting properties in topical application, by attenuated skin papillomagenesis[233], and inhibited tumour growth and metastasis via anti-angiogenesis activity[229].

The previously reported laboratory and *in vivo* animal studies provide substantial evidence that 6-gingerol is an effective inhibitor of the carcinogenic process. Additional studies on the determination of anticancer activity should ideally include human intervention trials to investigate its efficacy against human cancers and other diseases.

1.3.3. Other Applications of Ginger Rhizome

In addition to the biological activities already referenced in the previous topics, there are several applications in the traditional medicine for ginger rhizome that science has tried to explain. Migraine is an event of a neurological nature that can be accompanied by visual, gastrointestinal and/or premenstrual disorders. Several hypotheses have been proposed to give the possible abortive effect of ginger on migraine headaches, focusing on the receptors already associated in migraine therapy. Agents in use for migraine therapy modify the release of transmitters in the transmission of pain, block the sensitization of nerve fibers or block the mechanisms of inflammation that surround the wall of blood vessels. Considering that ginger is used in the *Ayurvedic and Tibb* medicine systems, its consumption has been

proposed to respond to the pathology, verifying that there was a reduction in the incidence of migraines[234]. So, the consumption of fresh or powdered ginger may have an effect on migraine headache and its use may also be helpful in childhood and juvenile headache, as there are no side effects associated with this rhizome.

6-gingerol was as well tested against the anti-writhing action. It was administered 10 mg/kg indomethacin and 12.5 mg/kg, 25 mg/kg and 50 mg/kg of 6-gingerol to rats, in order to test the effect of 6-gingerol in writhing – twist the body about, in response to pain, in acute discomfort[216]. Both the administration of indomethacin and 6-gingerol (25 mg/kg and 50 mg/kg) significantly reduced the number of writhing induced by 1% acetic acid for 10 minutes. Thus, 6-gingerol may have an analgesic effect.

1.3.4. Encapsulation of Ginger Compounds – State of the Art

A search in SCOPUS scientific search engine on "cyclodextrin" and "gingerols" affords four hits. Two of these results are out of the scope of inclusion complex isolation, since the CDs are used to purify the ginger components in chromatography stationary phases. The third hit is a patent update about atherosclerosis, with reference to ginger as an antioxidant claim-mechanism[235]. The fourth result, an article from 2007 entitled "Preparation optimization and characterisation of ginger oleoresin/β-cyclodextrin inclusion complex", has only the abstract available, allowing one to learn that the complexation process involved a mass ratio of β-CD:ginger oleoresin of 7:1 stirred for 1h at 65 ºC to afford an IC with a gingerol inclusion rate of 95%[236].

1.4. Purpose of the Work

Cyclodextrins are, as stated before, molecules that allow the encapsulation of various guests, affording a product with higher solubility and higher stability while the biological properties associated with the guest are maintained. In this work, we focus on two different types of guests be used, for the properties they present and for the interest in the scientific development of a regional product.

Cistus ladanifer is a plant that develops only in the Mediterranean region, with antioxidant and antimicrobial properties, present at labdanum, flavonoid extract and essential oil. Specifically, *Cistus ladanifer* essential oil exhibits antimicrobial effects against bacterial and fungal strains, which are important for cosmetic and skin-safe products. Thus, research into the essential oil *Cistus ladanifer* may allow obtaining a product with antimicrobial activity, which may be important for application under bacterial conditions of the skin, such as acne, a microbial-associated condition that strikes immensely among young people. Thus, the application of the essential oil to a cosmetic product would be of interest and, in order to avoid the risk of skin irritation and/or rashes, the inclusion of the essential oil into cyclodextrins appears to be a good solution. Keeping in mind also that the encapsulation of *Cistus ladanifer* essential oil may not occur with all components of the essential oil, the selective inclusion of certain compounds may lead to the emergence of new interesting properties.

Ginger also has a wide range of other properties. Although of Indian origin, it is widely distributed throughout the world, being used in traditional medicine with great appreciation. Considering the possible application of a ginger rhizome extract in the food, it is possible to emphasize the gastrointestinal, hypoglycemic and hypocholesterolemic activities, not excluding that it also has anti-inflammatory and antioxidant activity.

Therefore, molecular encapsulation increases the application in matrices of a hydrophobic character different from those of the host molecule.

2. Experimental

Next, it is presented the materials needed for the development of the work, as well as the protocols followed. With the protocols, the type of specific material required for the test is referenced.

2.1. Materials

Cistus ladanifer essential oil was acquired from Herdade de Vale Côvo, Mértola, Alentejo, an agrotourism and biological agriculture company. The extract containing gingerols was obtained from ginger rhizome acquired a local supermarket, as detailed in the subsection 2.2.5. below.

β-Cyclodextrin decahydrate (β-CD, Mr = 1316 g/mol) and γ-cyclodextrin heptahydrate (γ-CD, Mr = 1440 g/mol), from Wacker-Chemie, were kindly offered by Ashland Specialty Chemicals. The gingerols standard (Mr=310.10 g/mol), dissolved in ethanol analytically pure, was provide from previous work.

For the characterization methods and the biochemical assays, it was necessary potassium bromide (KBr) and acarbose solution, α-glucosidase enzyme solution, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), butylated hydroxyanisole (BHA), β-carotene, linoleic acid, 4-nitrophenyl-α-D-glucopyranoside solution, potassium persulfate ($K_2S_2O_8$) and tween 80, all from Sigma-Aldrich.

The following solvents (analytically pure) were used: acetone (from VWR), chloroform (Merck), deuterated chloroform (unknown brand), dichloromethane (unknown brand), dimethyl sulphoxide (VWR), ethanol (Panreão Química) and methanol (Fisher Scientific).

For the production of yogurt, Mimosa® fat milk and Activia® yogurt (Danone®) were used.

2.2. Methods

2.2.1. Instrumentation

The GC-MS analyses were performed on a CGMS (Gas Chromatograph Mass Spectrometer)-QP2010 Ultra equipped with autosampler AOC-20i, ion source of electronic

impact, an High-performance Quadrupole Mass Filter and a Agilent DB-5 ms column, with 30 m length, 0.25 mm diameter and 0.25 µm thickness. The initial temperature of the oven was 50 °C and the temperature in the injector was 250 ºC. The injector went into split mode (1:50). The run was at 69.4 kPa, with a total flow of 65.0 mL/min and a column flow of 1.22 mL/min. The linear velocity was 40.0 cm/s and the purge flow was 3.0 mL/min. The analysis proceeded at 50 ºC for 3 min, rising at 2 ºC per minute to 250 ºC, and maintained at that temperature for 10 minutes. The samples were dissolved in chloroform.

Mass spectrometry (ESI-MS) was performed on a Micromass Q-TOF-2 ™ mass spectrometer. The ionization of the sample was done in electrospray in positive mode, with 3 kilovolts in the needle and 30 volts in the cone.

Fourier Transformed Infrared Spectroscopy (FT-IR) spectra were collected in a Mattson Unican Instrument. 7000 galaxy series FT-IR 7000 spectrophotometer using KBr pellets, averaging 64 scans at a resolution of 2 cm^{-1}. According to this characterization method, the guests and the inclusion compounds were analyzed. For the guests, since there are two oily compounds, two extremely fine KBr pellets were made and the oil placed between the two.

Differential Scanning Calorimetry (DSC) was carried out in a power-compensated DSC, PerkinElmer Diamond DSC. Following a five-minute lat period at 20 ºC, samples were heated to 270 °C at a rate of 5 °C/min. Sample mass was of 2.653 mg of ginger acetone extract and 3.616 mg for the respective inclusion complex.

Liquid-fase ^{1}H NMR and ^{13}C NMR spectra were collected at 300 MHz using a Bruker Avance III. The samples were dissolved in deuterated chloroform in order to perform the assay, using tetramethylsilane (TMS) as standard.

Solid State $\{^{1}H\}$ ^{13}C CP/MAS NMR spectra were collected at 100.62 MHz on a Bruker AVANCE III 400 MHz (9.4 T) spectrometer, with a $\pi/2$ pulse, optimized at 3 milliseconds Contact for ^{1}H. Samples were run at 12 kHz with 4 seconds between scans.

Powder X-Ray Diffraction (PXRD) data was collected at room temperature in a Philips X'Pert MPD diffractometer with a copper radiation Kα1 = 1.540598 Å, equipped with a X'Celerator detector and in a Bragg-Brentano configuration (40 kV, 50 mA). The data was collected in pulses of 0.04º and ¼ and ½ slits, continuously in $3.5º \leq 2\theta \leq 50º$ interval. γ-CD·gingerols was also collected overnight with the following characteristics: pulses of 0.02º and slits ⅛ and ¼, in a continuous $3.5º \leq 2\theta \leq 50º$ interval.

Absorbance reading for the β-carotene, α-glucosidase inhibition and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assays, were collected on 96-well plates with a EON BIOTEK plate reader, with Gen 2.05.5 software, using the specific working wavelength of each assay.

The pH value of yogurts were measured with a glass electrode at 20 °C (pH electrode 50 14, Crison Instruments, S.A., Spain) properly calibrated.

The colour of manufactured yogurt was analyzed using a CM2300d spectrometer with Spectramagic NX software, both from KONICA MINOLTA. This portable spectrometer is specific for solid samples, so the assay for the yoghurt sample had to be slightly adapted. The samples were scattered on a watch glass and the equipment was placed on them. The portable spectrometer has a hole in which a sensor is located a few centimeters apart. The distance of the sample with the hole was taken into account so that the sample to be analyzed did not enter the hole. For the yogurt samples, the parameters L^*, a^* and b^* were obtained (n = {5, 10}).

2.2.2. *Cistus ladanifer* Essential Oil Physical Properties

Density: The essential oil was characterized in relation to its density: 300 μL of essential oil was weighed, yielding a mass of 289.8 mg. Thus, the density is 0.966 g/mL.

Molecular Weight: GC-MS analysis allowed to define a molecular mass for the essential oil, by analyzing the compounds which are in the composition of the essential oil and their amount. So, 10 μL of *Cistus ladanifer* essential oil was diluted in 1 mL of chloroform. The molecular weight of the essential oil is 143.685 g/mol with a 5.69% – this information will be developed in the subsection 3.1.1.

FTIR (KBr): ν (cm^{-1}) = 3466 m, 2957 vs, 2923 vs, 2871 s, 2834 m, 2731 w, 2725 w, 1790 sh, 1770 w, 1737 m, 1721 m, 1716 sh, 1713 sh, 1710 m, 1683 m, 1679 m, 1674 sh, 1661 sh, 1652 w, 1645 w, 1634 w, 1626 w, 1617 sh, 1516 w, 1506 w, 1495 sh, 1467 sh, 1463 sh, 1454 m, 1436 sh, 1418 w, 1411 w, 1383 m, 1375 m, 1365 m, 1336 w, 1328 w, 1313 sh, 1306 sh, 1300 w, 1281 sh, 1261 sh, 1245 m, 1204 w, 1179 w, 1164 w, 1145 w, 1125 w, 1112 w, 1100 sh, 1084 w, 1048 m, 1032 m, 1021 sh, 1014 sh, 997 sh, 953 w, 945 sh, 926 sh, 887 m, 876 sh, 857 w, 843 sh, 839 w, 823 sh, 816 w, 801 sh, 787 w, 772 w,

760 w, 746 w, 737 vw, 725 sh, 720 w, 716 sh, 711 vw, 698 vw, 685 w, 668 w, 655 sh, 649 w, 639 sh, 636 vw, 618 w, 611 w, 604 w, 597 sh, 591 w, 587 sh, 580 sh, 576 vw, 564 w, 543 w, 537 sh, 531 sh, 525 w, 516 w, 511 vw, 482 vw, 478 vw, 472 vw, 467 sh, 460 sh, 457 vw, 452 vw, 442 vw, 431 vw, 422 vw, 419 vw, 397 vw, 393 sh, 384 vw, 374 vw, 359 sh, 350 w, 340 w, 334 w, 327 w, 323 w, 319 sh, 312 sh.

2.2.3. Preparation of β-Cyclodextrin·*Cistus ladanifer* Essential Oil (β-CD·Cleo)

The formation of the inclusion complex occurred by co-precipitation. Typically, to a saturated aqueous solution of β-CD at 40 °C[237] (see Table 15) was added an equimolar amount of *Cistus ladanifer* essential oil dispersed in ethanol (1:3 proportion).

Table 15 – Experimental conditions for each encapsulation reaction with β-cyclodextrin.

Experiment	β-CD *(g)*	H_2O *(mL)*	Cleo *(μL)*	Yield
#1	0.5000	14.3	57	202.9 mg (36.4 %)
#2	1.0014	28.7	113	296.2 mg (26.7 %)
#3	2.0015	57.4	226	1 200 mg (54.1%)

Each solution was allowed to cool slowly for one day and then placed in the refrigerator for two hours. Later, it was centrifuged at 3500 revolutions per minute (rmp) for 30 minutes. The supernatant was withdrawn and the solutions stored in the refrigerator overnight. The solutions were then centrifuged again under the same conditions. The formed complexes were placed for four days in the presence of silica in order to withdraw the water still present in the sample.

FTIR (KBr): ν (cm^{-1}) = 3349 vs, 2927 w, 2992 w, 2363 vw, 2358 vw, 2352 sh, 2341 vw, 2338 sh, 2331 vw, 2324 sh, 2322 vw, 2307 sh, 1771 vw, 1748 sh, 1738 sh, 1732 vw, 1716 sh, 1707 vw, 1700 w, 1695 vw, 1683 vw, 1652 vw, 1646 w, 1635 w, 1627 vw, 1617 vw, 1576 vw, 1569 vw, 1558 vw, 1539 vw, 1532 vw, 1520 vw, 1516 vw, 1506 vw, 1493 sh, 1488 sh, 1480 sh, 1456 w, 1446 w, 1436 w, 1430 w, 1423 sh, 1417 w, 1409 sh, 1384 w, 1369 w, 1333 w, 1302 w, 1247 w, 1220 w, 1158 s, 1125 sh, 1112 sh, 1102 m, 1080 s, 1057 s, 1029 vs, 1004 m, 945 w, 938 w, 905 vw, 888 vw, 861 vw, 840 sh, 758 w, 703 w, 695 sh, 668 w, 652 w, 608 sh, 592 w, 575 w, 528 w, 502 w, 475 w, 443 w, 418 sh,

413 w, 398 sh, 357 w, 335 vw, 329 vw, 323 vw, 318 sh, 315 vw, 310 vw, 302 vw, 298 vw, 295 vw, 290 vw, 286 vw.

^{13}C CP/MAS NMR (12 kHz, 25 °C, ppm): δ = 192.0, 180.0 (guest, C=O), 145.3, 128.3, 126.0 (guest, aromatic region), 116.6, 110.7 (guest), 103.6, 102.8 (β-CD, C_1), 83.8, 81.7, 80.7, 80.1 (β-CD, C_4), 74.3, 73.6, 72.6, 70.7 (β-CD, $C_{2,3,5}$), 61.0, 60.0 (β-CD, C_6), 50.6, 48.6, 46.6, 41.3, 41.3, 37.1, 31.4, 27.4, 23.9, 21.1 (all guest).

2.2.4. Preparation of γ-Cyclodextrin·*Cistus ladanifer* Essential Oil (γ-CD·Cleo)

The encapsulation of *C.ladanifer* essential oil in γ-cyclodextrin followed the same procedure, taking into account that the solubility of γ-CD at 40 °C is 460 mg/g of water[237].

Table 16 – Experimental conditions for each encapsulation reaction with γ-cyclodextrin.

Experiment	γ-CD *(g)*	H_2O *(mL)*	Cleo *(μL)*	Yield
#1	0.5000	1.1	52	245.1 mg (44.6 %)
#2	1.0014	2.2	105	714.5 mg (71.3 %)
#3	2.0015	4.4	210	1 786.9 mg (80.9%)

The inclusion complexes were isolated following the procedure described in 2.2.3.

FTIR (KBr): ν (cm^{-1}) = 3382 vs, 2926 w, 1734 w, 1772 sh, 1717 w, 1706 sh, 1700 w, 1696 w, 1684 w, 1675 sh, 1670 sh, 1646 w, 1639 w, 1632 w, 1624 sh, 1618 w, 1458 w, 1437 sh, 1416 w, 1405 sh, 1383 w, 1373 w, 1366 sh, 1337 w, 1303 w, 1246 w, 1201 w, 1159 s, 1127 sh, 1106 m, 1080 s, 1058 sh, 1054 s, 1027 vs, 1002 s, 981 sh, 941 w, 865 w, 839 sh, 815 sh, 794 sh, 761 w, 724 sh, 705 w, 686 w, 668 w, 660 w, 656 w, 651 w, 644 w, 611 sh, 596 sh, 581 m, 565 sh, 531 w, 512 sh, 501 w, 496 w, 490 w, 479 w, 468 w, 463 sh, 457 w, 448 sh, 443 w, 437 w, 432 w, 427 sh, 418 w, 411 w, 399 w, 391 sh, 384 w, 375 w, 364 sh, 358 w, 351 w, 342 sh, 334 w, 328 w, 322 w, 319 sh, 311 w, 304 w, 300 sh, 296 w, 290 sh.

^{13}C CP/MAS NMR (12 kHz, 25 °C, ppm): δ = 191.3, 180.0 (guest C=O), 144.6, 129.3, 126.7 (guest, aromatic region), 116.0, 110.0 (guest),105.2, 103.9 (γ-CD, C_1), 82.6, 82.1, 80.8 (γ-CD, C_4), 74.3, 74.1, 73.6, 73.2, 72.8, 71.9, 71.2 (γ-CD, $C_{2,3,5}$), 60.3 (γ-CD, C_6), 50.6, 48.6, 47.3, 41.3, 37.4, 31.4, 26.6, 23.9 (all guest).

2.2.5. Evaluation of the Included Components

In order to understand the encapsulation occurred, the hosts were extracted using chloroform, the cyclodextrin precipitated and the liquid was filtered, using nylon filters of 0.45 μm porosity, so as to be injected into GC-MS for analysis. For this purpose, 101.2 mg of β-CD·*Cistus ladanifer* and 60.235 mg of γ-CD·*Cistus ladanifer* were precipitated with 2 mL of chloroform, as use of chloroform allowed the release of the compounds that were encapsulated. These samples were analyzed by GC-MS, with the same characteristics referenced in subsection 2.2.2.

2.2.6. Extraction and Purification of Ginger Extract from the Ginger Rhizome

Chopped ginger (600 g) was extracted with acetone for 48 hours, under stirring on an orbital shaker at 90 rpm. The suspension was then filtered with gauze and the mother liquors were evaporated at reduced pressure. The oil was redissolved in water and extracted with mixtures of dichloromethane:methanol (3 x 100 mL), with growing polarities from 5% to 15%. After evaporation of the solvents the residue was redissolved with 50 mL of dichoromethane and rotavaped with 3 grams of silica gel until a solid residue was achieved. The silica column was prepared with dichloromethane and the powdered sample was added to the top of the column. At this point, elution with a solvent gradient, ranging from 1 to 5 % methanol in dichloromethane and collection of a yellowish fraction gave rise to an impure fraction of ginger acetone. Further purification over preparative TLC plates (sample diluted with dichloromethane and eluted with chloroform) and evaporation of the solvents under reduced pressure provided 288.9 mg of pure ginger extract.

2.2.7. Ginger Extract Physical Characterization

Mass Spectrometry: Mass spectrometry analysis allowed to define a molecular mass for the essential oil, by analyzing the compounds which are in the composition of the essential oil and their amount. The molecular weight of ginger extract is 314.71 g/mol. This information will be developed in the subsection 3.2.1.2.2. – Table 25.

This sample contained other compound differing from 6-gingerol (Figure 9), as 8- and 10-gingerol. In order to simplify the numerical assignment of the hydrogens and carbons as the assignment of all compounds differ from IUPAC identification, it was used letters instead of numbers, starting in the aromatic ring – this alteration made possible the identification of the carbonyl (C_j) and carbon identifying the gingerol (C_l) with the same letter.

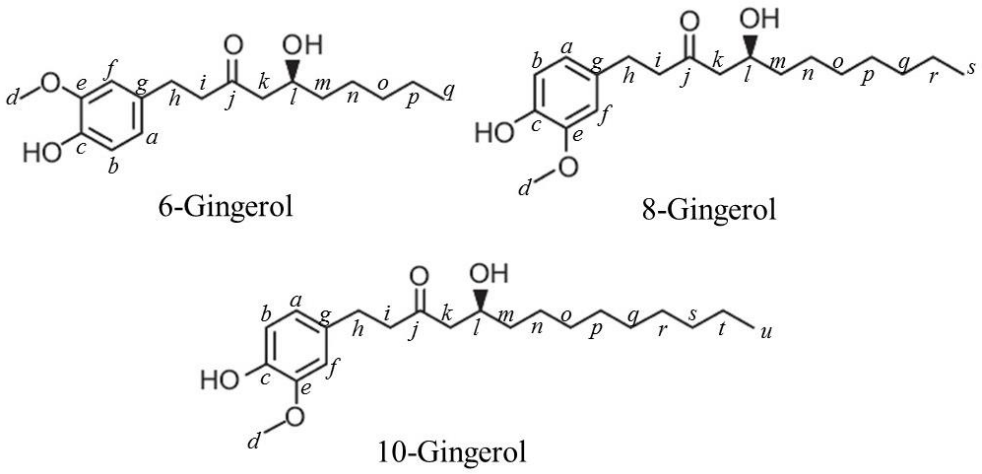

Figure 9 – Structure of 6-, 8- and 10-gingerol, with the letter identification used for this work.

FTIR (KBr): ν (cm^{-1}) = 3461 vs, 2954 vs, 2951 vs, 2942 sh, 2937 sh, 2929 vs, 2926 sh, 2921 sh, 2918 sh, 2884 s, 2876 sh, 2870 sh, 2857 s, 1736 w, 1707 s, 1704 s, 1698 s, 1693 s, 1681 m, 1671 sh, 1665 w, 1659 w, 1649 w, 1643 sh, 1632 sh, 1619 sh, 1610 sh, 1602 m, 1594 sh, 1573 w, 1556 w, 1562 w, 1555 sh, 1551 sh, 1547 w, 1515 vs, 1493 w, 1464 s, 1453 s, 1439 sh, 1431 s, 1407 m, 1383 sh, 1375 sh, 1370 s, 1359 sh, 1271 vs, 1236 s, 1208 s, 1187 sh, 1152 s, 1131 sh, 1123 s, 1087 m, 1034 s, 992 sh, 927 w, 894 w, 853 w, 824 sh, 813 m, 796 m, 724 w, 706 w, 671 w, 665 sh, 624 w, 557 m.

^{1}H NMR (20 ºC, ppm): δ = 6.84, 6.81 (H-*f*), 6.68, 6.65, 6.64 (H-*a*, H-*b*), 5.49 (*c*-OH), 4.02 (*l*-OH), 3.87 (*d*-CH_3), 2.96, 2.93 (H-*l*), 2.89, 2,87, 2.86, 2.84, 2,81, 2,76, 2.75, 2.73, 2.72, 2.70 (*i*-CH_2-*h*, *g*-CH_2-*a*), 2.61, 2.60, 2.55, 2.52, 2.49, 2.46, 2.44 (*k*-CH_2-*j*), 1.31, 1.28, 1.26 (*p*-(CH_2)*n*-*m*), 0.91, 0.89, 0.88, 0.86 (*q*-CH_3).

^{13}C NMR (20 ºC, ppm): δ = 211.5 (C_j), 146.4 (C_e), 144.0 (C_c), 132.6 (C_g), 120.7 (C_a), 114.4 (C_b), 111.0 (C_f), 67.7 (C_d), 55.9 (C_l), 49.3 (C_k), 45.4 (C_i), 36.4 (C_m), 31.7 (C_h), 29.3 (C_o), 25.1 (C_n), 22.6 (C_p), 14.0 (C_q).

2.2.8. Preparation of γ-Cyclodextrin·Gingerols

The formation of the inclusion complex, in which the host was the gingerols, proceeded in the same way as the formation of the referenced complexes. Starting with 140.3 mg of gingerols for a stoichiometry of 1:1, 634.5 mg of γ-cyclodextrin was dissolved in 1.38 mL of water at 40 °C. Gingerols were diluted in 800 μL of ethanol and associated with the γ-CD solution. As the gingerols extract appeared to be more hydrophobic than *Cistus ladanifer* essential oil, the γ-CD·gingerols was left over night, about eighteen hours, at 40 °C in order to stabilize. Several aggregates of gingerols extract appeared, whereby 1 mL of ethanol and 500 μL of methanol were added. This addition allowed to decrease the diameter of the gingerols extract aggregates. The final solution was allowed to cool slowly for four days. Then, the solution was centrifuged at 3500 rmp for 50 minutes and the supernatant was stored. The formed complex was placed in wristwatch glass overnight in the presence of silica. It was obtained 721.5 mg of complex, yield = 93.1 %.

*FTIR (KBr):*ν (cm^{-1}) = 3366 vs, 2946 sh, 2926 m, 2902 sh, 2851 sh, 1719 w, 1707 w, 1703 w, 1698 m, 1693 m, 1687 sh, 1681 sh, 1677 w, 1672 w, 1664 sh, 1659 sh, 1657 w, 1649 w, 1643 sh, 1632 w, 1625 sh, 1620 w, 1613 w, 1602 m, 1579 sh, 1572 w, 1565 w, 1563 w, 1555 w, 1551 w, 1547 w, 1543 sh, 1535 sh, 1529 sh, 1524 sh, 1516 m, 1503 w, 1492 sh, 1468 sh, 1460 w, 1453 w, 1431 sh, 1421 m, 1415 m, 1383 m, 1374 sh, 1351 sh, 1336 m, 1300 w, 1273 m, 1255 w, 1240 m, 1200 w, 1158 s, 1126 sh, 1105 m, 1079 s, 1051 sh, 1026 vs, 1001 s, 942 m, 935 sh, 917 sh, 890 w, 870 sh, 860 w, 810 sh, 793 sh, 761 w, 704 m, 608 m, 580 m, 554 sh, 529 m, 476 w, 449 sh, 442 sh, 412 w, 400 sh, 390 w, 362 w, 358 w, 346 w, 338 sh, 331 w.

^{13}C CP/MAS NMR (12 kHz, 25 °C, ppm): δ = 210.0 (gingerols, C_j), 147.5 (gingerols, C_e), 144.6 (gingerols, C_c), 132.5 (gingerols, C_g), 115.4 (gingerols, C_b), 111.7 (gingerols, C_f), 105.2, 104.1 (γ-CD, C_1), 82.9, 82.6, 82.3, 81.1 (γ-CD, C_4), 74.6, 74.1, 73.7, 73.2, 73.0, 72.6, 71.9, 71.2 (γ-CD, $C_{2,3,5}$), 67.5 (gingerols, C_d), 60.8, 60.4 2 (γ-CD, C_6), 56.7 (gingerols, C_l), 50.0 (gingerols, $C_{k/i}$), 36.7 (gingerols, C_m), 31.7 (gingerols, C_h), 29.2 (gingerols, C_o), 25.0 (gingerols, C_n), 22.5 (gingerols, C_p), 14.2 (gingerols, C_q).

2.2.9. Yogurt Manufacture

Yogurt is a fermented dairy product widely consumed as functional food due to its good taste and nutritional properties, as it is rich in potassium, calcium, protein and vitamin B. Furthermore, it is an excellent vehicle to deliver probiotics to consumers[238]. It is the food produced by a characterised bacterial culture that contains the lactic acid-producing bacteria, *Lactobacillus bulgaricus* and *Streptococcus thermophilus*[239].

The yogurts were produced in laboratory, reproducing as similar as possible the conditions provided by a yoghurt maker. For this purpose, fat milk and yogurt were used, as the yoghurt provided the necessary bacterial culture to give the enzymatic changes for the yogurts production. Table 17 presents the quantities added in each flask in order to perform the assay.

Table 17 – Conditions for yogurts production.

		$m_{\text{compound added}}$ /mg	**m_{yogurt} /mg**	**v_{milk} /mL**
Simple Yogurt	#1	—	654.4	5.0
	#2	—	632.3	5.0
	#3	—	617.5	5.0
Gingerols Yogurt	#4	38.0	641.5	5.0
	#5	38.2	646.2	5.0
	#6	24.1	621.8	3.0
γ-CD·Gingerols Yogurt	#7	50.0	626.0	5.0
	#8	50.0	626.0	5.0
	#9	50.6	640.9	5.0

Note:

$m_{\text{compound added}}$ /mg – mass of gingerols/γ-CD·gingerois added to each vial in mg;

m_{yogurt} /mg – mass of yogurt added to each vial in mg;

v_{milk} /mL – volume of fat milk added to each vial, in mL.

The nine flasks were kept for 18 hours in a 40 °C bath, removed and stored in the refrigerator, similar to the conditions maintained during the production in a yoghurt maker. Note that although literature data often refers to the use of powder milk in yogurts formulations, a preliminary experiment in this work showed that this ingredient was unnecessary for the obtaining of a solid yogurt.

2.2.10. Biochemical Assays

The following tests were performed under gingerols and γ-CD·gingerols samples. For the ABTS assay, the gingerols and γ-CD·gingerols were previously applied in yogurts. The results and discussion are presented in subsection 3.4.

2.2.10.1.β-Carotene Assay

β-Carotene Solution and Blank Solution: First, a β-carotene solution (2 mg/mL) was prepared in chloroform. Next, a 1 mL of β-carotene solution was added to a round-bottom flask, where previously 1 g of tween 80 was weighed. Then, the chloroform was removed under reduced pressure and 40 ºC. Using the balance, it was added 50 mg of linoleic acid and the solution was homogenized. At last, a total of 100 g of water was slowly added to the previous mixture – the total removal of the chloroform from β-carotene solution led to a clean emulsion. It was also prepared a blank, which the 1 mL of β-carotene solution was replaced by 1 mL of chloroform.

Standard Solution: A solution of BHA (0.5 mg/mL) was also prepared: about 10 mg BHA was weight and 1 mL of ethanol was added in order to dissolve the BHA. Then, the volumetric flask of 20 mL was completed with water. This solution was diluted to a 2.5 µg/mL solution and seven standard solutions of BHA were prepared from this one – 0.8 µg/mL, 1.0 µg/mL, 1.2 µg/mL, 1.4 µg/mL, 1.6 µg/mL, 1.8 µg/mL and 2.0 µg/mL.

Samples Solution: For this assay, γ-cyclodextrin, gingerols extract and the encapsulation product were analyzed. The three samples solution were in the concentrations range of 38.6 µM to 101.8 µM. Gingerols were previously dissolved in ethanol, since they are not soluble in water.

Method:

On a 96-well plate, 40 µL of BHA or samples were applied, following by 200 µL of β-carotene or blank solution. The plates were incubated at 50 ºC for two hours and the absorbance was read at 470 nm. Values were compared with readings collected prior to incubation (baseline reading).

The percentage of inhibition is calculated according to the equations below:

$$\Delta \text{ABS}^{c=0} = \left(\text{ABS}_{\beta c}^{c=0} - \text{ABS}_{\text{Br}}^{c=0}\right)^{0h} - \left(\text{ABS}_{\beta c}^{c=0} - \text{ABS}_{\text{Br}}^{c=0}\right)^{2h} \quad - \textit{Equation 1}$$

$$\Delta \text{ABS}^{c=?} = \left(\text{ABS}_{\beta c}^{c=?} - \text{ABS}_{\text{Br}}^{c=?}\right)^{0h} - \left(\text{ABS}_{\beta c}^{c=?} - \text{ABS}_{\text{Br}}^{c=?}\right)^{2h} \quad - \textit{Equation 2}$$

$$\%\text{ of inhibition} = \frac{\Delta \text{ABS}^{c=0} - \Delta \text{ABS}^{c=?}}{\Delta \text{ABS}^{c=0}} \times 100 \quad - \textit{Equation 3}$$

Table 18 explains the several abbreviations used in the equation.

Table 18 – Resume table on the meaning of the abbreviation of the equations.

Abbreviation	**Meaning**
$\text{ABS}_{\beta c}^{c=0/?}$	Absorbance at concentration 0 or other concentration, with β-carotene and acid linoleic emulsion.
$\text{ABS}_{\text{Br}}^{c=0/?}$	Absorbance at concentration 0 or other concentration, with blank and acid linoleic emulsion.
$\left(\text{ABS}_{\beta c}^{c=0} - \text{ABS}_{\text{Br}}^{c=0}\right)^{0h}$	Differential absorbance in the beginning of the assay.
$\left(\text{ABS}_{\beta c}^{c=0} - \text{ABS}_{\text{Br}}^{c=0}\right)^{2h}$	Differential absorbance after 2 hours.

2.2.10.2.α-Glucosidase Assay

The α-glucosidase assay protocol was adapted from the existing protocol to rapidly understand the response of this enzyme to the sample. 4-nitrophenyl α-D-glucopyranoside was used as the substrate. This compound develops a yellow colour by hydrolysis, upon clivage. Acarbose, i.e. an anti-diabetic drug used to treat type 2 diabetes, was used as the reference inhibitor.

A high concentration of gingerols – c = 1.03 x 10^{-3} mol/L, i.e., 0.325 mg/mL – was prepared in a solution of 40% DMSO and maintained frozen up to use. The 4-nitrophenyl α-D-glucopyranoside solution was diluted to 6 mM and the enzyme stock solution (1 U/mL) was diluted so that its activity was at 0.04 U/mL.

In a 96-well plate, it was added 50 µL of 40% DMSO solution, 50 µL of 4-nitrophenyl α-D-glucopyranoside solution and 100 µL alpha-glucosidase enzyme, in triplicate, and 50 µL of 40% DMSO solution, 50 µL of 4-nitrophenyl α-D-glucopyranoside solution and 100 µL of gingerols solution also in triplicate. The reaction kinetics were measured by measuring the absorbance at 405 nm, minute by minute, during 20 minutes.

2.2.10.3.ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) Assay

ABTS Solution: 38 mg of ABTS were dissolved in 10 mL of H_2O and 6.6 mg of potassium persulfate ($K_2S_2O_8$) was added. The solution was left in the dark for 12 hours and divided in aliquots. From one of this aliquots, it was retired 1 mL and diluted with 40 mL of ethanol:water 1:1, in order to the absorbance at 734 nm reached between 0.750-0.800.

Standard Solution: A solution of ascorbic acid (1.42 mmol/L) was prepared: about 5 mg ascorbic acid was weight and dissolved in 20 mL of ethanol:water 1:1. Several dilution were prepared – 21.2 µM, 42.6 µM, 85.2 µM, 127.8 µM, 170.3 µM, 212.9 µM and 255.5 µM.

Extracts Solutions and Dilutions: Extracts were obtained from the yogurts. To 1 mL of ethanol was added to 1 g of yoghurt, and the sample was centrifuged for 30 minutes at 3500 rpm. The supernatant was collected, whereby the extract was obtained. Dilutions were established for the extracts, using ethanol:water 1:1 – Table 19.

Table 19 – Dilution applied for each sample.

		Dilution factors
Simple Yogurt	#1	4, 16, 28, 40
	#2	4, 16, 28, 40
	#3	4, 16, 28, 40
Gingerols Yogurt	#4	16, 28, 40, 60
	#5	16, 28, 40, 60
	#6	16, 28, 40, 60
γ-CD·Gingerols Yogurt	#7	4, 16, 28, 40
	#8	4, 16, 28, 40
	#9	4, 16, 28, 40

Method:

40 µL of standard or sample and 200 µL of ABTS were added to each well (in triplicate). The microplate was left in the dark for 20 min and then the absorbance was read at 734 nm. Using only four dilutions of the samples, it is applied the standard solutions calibration line, in order to define the concentration in ascorbic acid equivalents.

3. Results

As previously explored on Introduction section, cyclodextrin encapsulation of plant essential oils and lipophilic extracts is a good method to help stabilise their volatile components, protect light-sensitive compounds and increase their compatibility with aqueous formulations. For the encapsulation to be successful, the cyclodextrin must have a cavity adequate to the size of the molecules to encapsulate.

3.1. Inclusion Complexes of *Cistus ladanifer* Essential Oil

Prior to inclusion, the composition of *Cistus ladanifer* essential oil was investigated using GC-MS. Following, inclusion was attempted using two hosts: β- and γ-cyclodextrin, in order to understand if there is a selective encapsulation. The inclusion complexes were studied by infrared vibrational spectroscopy, solid state carbon 13 nuclear magnetic resonance ($\{^{1}H\}$ ^{13}C CP-MAS NMR) and powder x-ray diffraction (PXRD).

3.1.1. *Cistus ladanifer* Essential Oil GC-MS

The GC-MS analyser is connected to a data base of compounds, listed in the program, GCMS Postrun Analysis, which allows assigning most of the peaks. For those with various matching compounds, assignment relied on a comparison with the different components of *Cistus ladanifer* essential oil described in the literature. The graphical representation of the GC-MS results for *Cistus ladanifer* essential oil is presented in the Appendix A. Results are presented in Table 20 and Appendix B.

These two tables differed in the quantity of presented data. In Table 20, there are all the identified compounds with a percentage of area superior to 1%, while, in Appendix B table, there are all the compounds regardless the percentage of area. In this table, for the non-bibliographic identified compounds, the identification was established with similarity superior to 85% or, when this requirement was not obtained, it was tried to define the molecular weight.

Table 20 – Composition of Cleo. In this table, the majority components were selected, showing the retention time, compound name, molecular mass and peak area.

Retention Time	Compound Name	% Area	Molecular Weight
7.439	Tricyclene	1.67	136
7.947	α-Pinene	30.12	136
8.667	Camphene	8.58	136
8.872	Verbanene	0.94	134
12.675	*p*-Cymene	4.44	134
12.916	Limonene	1.93	136
13.058	Eucalyptol	0.95	154
13.293	2,2,6-Trimethylcyclohexanone	4.37	140
14.685	δ-3-Carene	1.43	136
19.060	α-Campholenal	1.67	152
19.844	*trans*-Pinocarveol	5.77	152
21.302	Pinocarvone	0.97	150
21.939	Borneol	1.88	154
22.171	Isopinocamphone	1.22	152
22.522	4-Terpineol	1.15	154
23.513	(-)-Myrtenal	0.80	150
29.416	Bornyl acetate	8.31	196
40.262	Aromandendrene	0.98	204
48.270	Viridiflorol	2.70	222

The average molecular weight (Mw) can be calculated by weighing the contribution of the components presented in Table 20, being thus estimated at 143.685 g/mol. Note that only 5.69% of the components remain unidentified and that these are not expected to significantly alter the Mw of *Cistus ladanifer* essential oil because they are most likely structural isomers with the same empirical formula (see table in Appendix B).

3.1.2. Cyclodextrin·*Cistus ladanifer* Inclusion Complexes

Cistus ladanifer inclusion complexes were prepared by co-precipitation, working close to the saturation for the host to favour the precipitation of the complex. Several experiments were conducted for each CD, as detailed in Tables 15 and 16 of the Experimental chapter. It is noteworthy that the yields for γ-CD·Cleo increased as larger quantities of the starting materials were used, but the same was not achieved in the case of β-CD·Cleo.

The complexes were characterized by Fourier transform infrared spectroscopy (FTIR), {^{1}H} ^{13}C CP/MAS NMR and powder X-ray diffraction. Information on the components of the essential oil that were included into the cavity of the CD was obtained from GC-MS, after the dissociation of an aliquot of the IC by selective dissolution of the guest into chloroform, in which the CDs are not soluble. Two cyclodextrins have different cavity size, meaning that β-CD may prefer to include components of smaller dimension while the γ-CD will include both smaller and larger components of the oil. The CG-MS analysis helps us understand selectivity associated with the process of encapsulation.

3.1.3. *Cistus ladanifer* Encapsulated Compounds Analysed by GC-MS

In order to examine the guest composition, the complexation process was reversed, precipitating cyclodextrin in chloroform, as described in subsection 2.2.5. It was possible to obtain a liquid which contained the guest composition and was analysed by GC-MS. In Table 21, there is the identification performed by GCMS Postrun Analysis program and the defined composition of the essential oil.

Table 21 – Composition of Cleo guest in β- and γ-CD complexes. Guest were analyzed by GC-MS and identification was made using the program and Cleo composition previously reported. For both complexes, it was possible to identify 76.38% of its composition.

Retention Time	**Compound Name**	**% Area of β-CD·Cleo guest**	**% Area of γ-CD·Cleo guest**
7.947	α-Pinene	0.84	46.43
8.667	Camphene		10.31
12.675	*p*-Cymene		2.36
13.293	2,2,6-Trimethylcyclohexanone		3.05
19.844	*trans*-Pinocarveol		4.85
29.416	Bornyl acetate	7.54	9.11
30.201	Myrtenyl acetate	1.10	
35.112	Copaene	1.48	
37.765	Caryophyllene	0.81	
37.983	(+)-Aromadendrene	2.10	
40.262	Alloaromadendrene	5.67	
42.828	α-Muurolene	1.14	
44.009	δ-Cardinene	4.63	
46.865	(L)-Ledol	11.18	
47.508	Caryophyllene oxide	1.79	
48.846	Epiglobulol	38.1	

The first observable difference between the GC-MS of pure and β-CD·encapsulated *Cistus ladanifer* essential oil is the almost complete absence of peaks in the retention time interval 4 to 30, while there are more components peaking within this interval in the case of γ-CD inclusion. Also noteworthy is a dramatic decrease in the number of peaks for both ICs, which is due to a selective inclusion, causing some compounds not to be included in the complexes. The different cavity size of the cyclodextrins allowed obtaining two ICs with different composition for the guest. Regardless of the cyclodextrin used, the essential oil composition is not maintained. β-CD encapsulated compounds of higher molecular weight and with high retention times, while γ-CD encapsulated compounds of lower molecular weight. The ICs are thus expected to have biological properties and activities different from those of the pure oil.

3.1.4. Fourier Transform Infrared Vibrational Spectroscopy Analysis (FT-IR)

An IR can be interpreted as a fingerprint of the sample and it is also possible to have information on the molecular structure, since bonds have a spectrum zone in which they manifest. The spectra of β-CD·Cleo and γ-CD·Cleo (Figure 10, green and blue traces, respectively) feature a great similarity between both of them, due to the similar molecular structure of the cyclodextrins. So, the most informative bands are those ascribed to the contribution of the essential oil. The collected data for the guest alone (Figure 10, orange trace) as well as for the inclusion complexes should be analyzed by comparison in order to understand what differs between them.

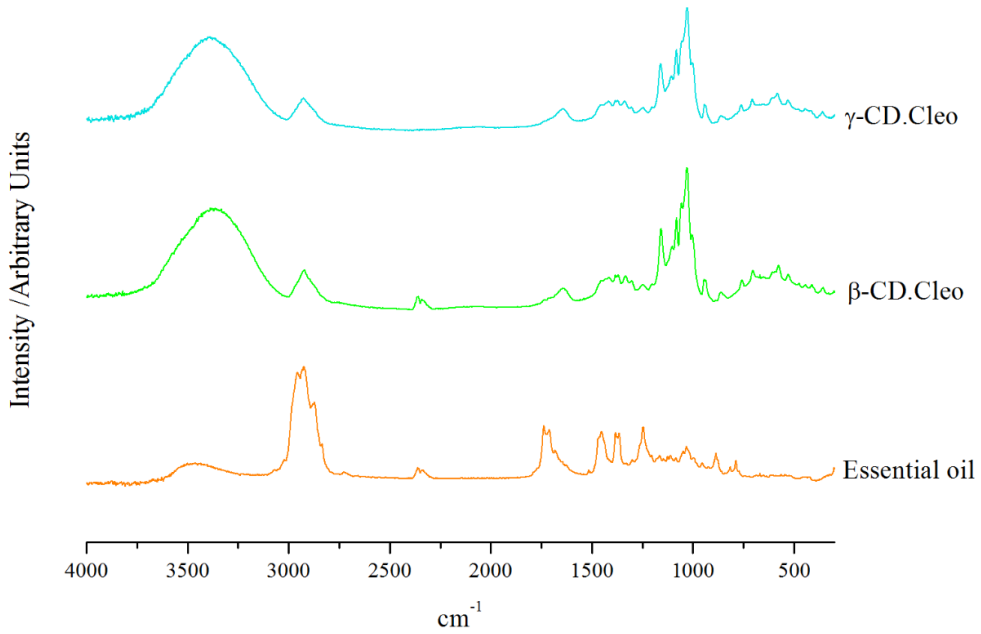

Figure 10 – FT-IR of Cistus ladanifer essential oil and its β- and γ-cyclodextrin inclusion complexes.

Around 3466 cm^{-1}, there is the contribution of the OH groups in the three samples[240], even though the pure oil has a much lower band as expected. In the range of 2800 to 2960 cm^{-1}, the pure essential presents several peaks with relative intensities between medium and very strong, ascribed to asymmetric ν(CH) from CH_2 and CH_3 groups[240]. The complexes have only one broad band centered at 2927 cm^{-1} for β-CD·Cleo and 2926 cm^{-1} for γ-CD·Cleo. An expansion of the region between 800 and 1800 cm^{-1} is present in Figure 11. Table 22 shows a summary of the most relevant bands.

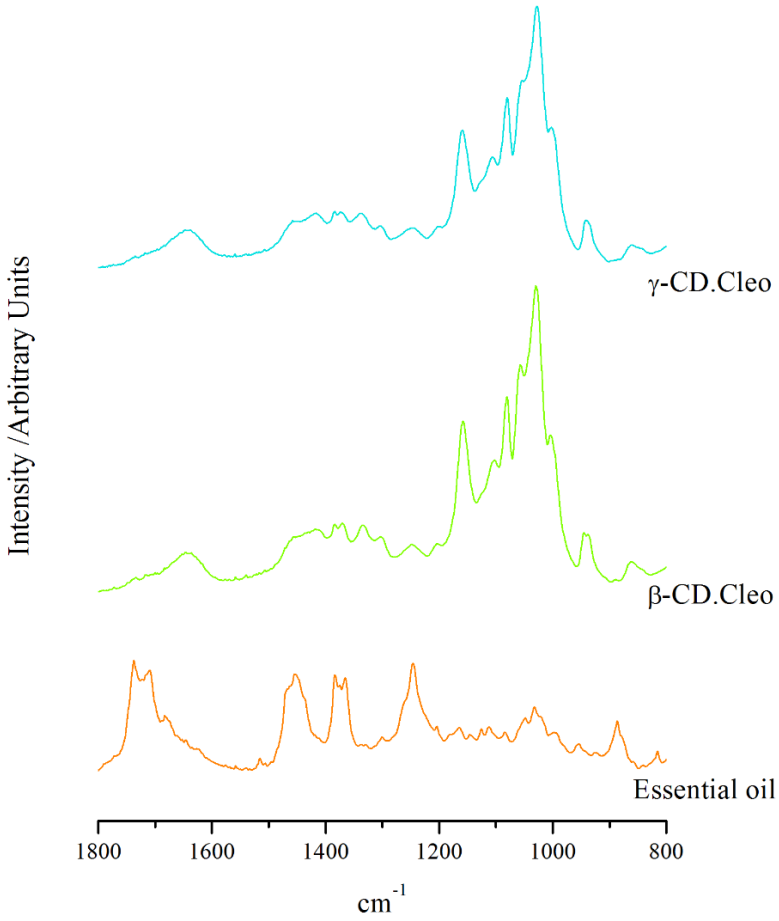

Figure 11 – Selected region of the spectra of Cistus ladanifer essential oil β-CD·Cleo and γ-CD·Cleo.

Table 22 – Selected bands of pure Cistus ladanifer essential oil and its inclusion complexes.

Cleo	**β-CD·Cleo**	**γ-CD·Cleo**	**Approximate Description**[240]
1738, 1716, 1710	1738, 1716, 1707	1734	ν(C=O) ester carbonyl[a]
1365	1369	1366	ω[b](C-H)
887	888	885	δ(C-O-C)

Note:

[a] – The observation of the C=O stretch in *C. ladanifer* oil is associated, according to Carrión-Prieto et al[240], with the presence of triglycerides in the sample;

[b] – ω is synonym of out of plane bending (wagging).

The presence of bands around 1700 cm^{-1} is indicative of the presence of triglycerides in the essential oil, which were also included into the two hosts. These were not identified using GC-MS, since their molecular weight is too high when compared to the remaining compounds of the oil. It is to be further noted that the presence of these compounds in the

inclusion complex has no advantage, since these have no relevant biological activity and are competing with the active components of the oil for space inside the CDs

3.1.5. Solid State {^{1}H} ^{13}C CP-MAS NMR

The {^{1}H} ^{13}C CP-MAS NMR spectra β-CD, β-CD·Cleo, γ-CD and γ-CD·Cleo are depicted in the Figure 12. Green traces were used for β-CD samples and blue for γ-CD. For the inclusion complexes, lighter shades were used, leaving the darker ones for the cyclodextrin alone.

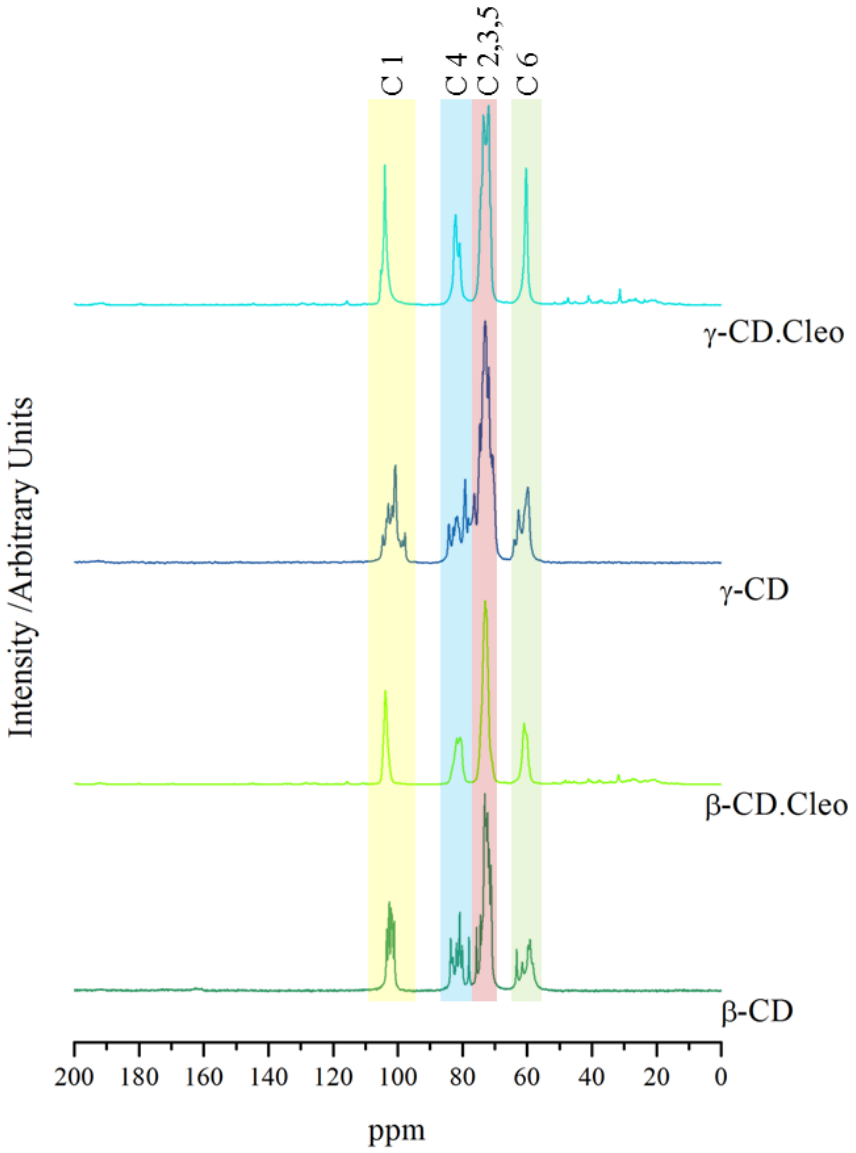

Figure 12 – ^{13}C {^{1}H} CP/MAS NMR analisys of β- and γ-cyclodextrins and their inclusion complexes with Cistus ladanifer essential oil, with the assignments according to the literature[241].

The most notable feature of the cyclodextrins and inclusion complexes is the modification of the multiplicity of the signals. While in the single cyclodextrins graphs, the signals were multiplied, for the same chemical shifts, in the graphs of the inclusion complexes, the signals are presented with lower multiplicity. In β-CD·Cleo, C2,3,5, C4 and C6 signals have double multiplicity, while carbons C1 appear as a single resonance. For γ-CD·Cleo, carbon 6 has simple multiplicity, whereas the remaining carbons present double or triple multiplicity. The change in multiplicity of the cyclodextrins' signals is due to the channel structure and the presence of a guest inside of the inclusion complexes, which induce CDs to adopt a more symmetrical conformation.

The spectra also present small resonances ascribed to the guest carbons. Given the complexity of the multicomponent sample and knowing that several compounds were included, it is possible to expect that the major compounds of each complex will be the contributors to the peaks identified. Thus, ^{13}C NMR data of α-pinene, camphene, ledol and epiglobulol were used to proceed to an identification of the remaining signals – Table 23.

Table 23 – Assignment of ^{13}C RMN identified carbons with the major components identified by GC-MS, units in ppm.

β-CD·Cleo	**γ-CD·Cleo**	**α-Pinene**[242]	**Camphene**[243]	**Ledol**[244 245 246]	**Epiglobulol**[244]
192.0	191.3	*possible tryglicerides not identified in GC-MS*			
180.0	180.0	*sppining sidebands from CDs influence*			
145.3	144.6	144.51 (C_1)			
128.3	129.3			*n. i.*	
126.0	126.7			*n. i.*	
116.0	116.0	116.14 (C_2)			
110.7	110.0			*n. i.*	
50.6	50.6			*n. i.*	
48.6	48.6		48.3 (C_4)		
46.6	47.3	47.24 (C_3)	47.0 (C_1)		
41.3	41.3		41.3 (C_3)	40.9 $(C_5)^+$, 40.8 $(C_5)^§$	42.9 (C_9)
37.4	37.4		37.4 (C_7)	38.4 $(C_4)^{+*§}$, 37.8 $(C_9)^*$	37.6 (C_5)
31.4	31.4	31.53 (C_6) 31.35 (C_7)		32.1 $(C_{14})^*$, 30.6 $(C_{14})^+$ 30.5 $(C_{14})^§$	31.2 (C_{14})
27.4				28.7 $(C_{12})^+$ $(C_{13})^§$	28.8 (C_{12}), 27.1 (C_7)
	26.6	26.43 (C_8)	25.9 (C_{10})	25.8 $(C_2)^*$, 25.1 $(C_7)^+$	26.6 (C_2)
23.9	23.9		23.9 (C_5)	23.5 $(C_6)^{+§}$	
21.1				22.3 $(C_6)^*$, 20.4 $(C_8)^+$	20.6 (C_{11})

Note: $^+$ – Data from Bombarda et al.[244], * – Data from Wu et al.[245], § – Data from Kaplan et al.[246];
n. i. – not identified.

3.1.6. Powder X-Ray Diffractograms of *Cistus ladanifer* Inclusion Complexes

Inclusion complexes were studied by powder X-rays diffraction, since the formed powders were microcrystalline. Figure 15 shows the diffractograms of β-CD (medium green), β-CD·Cleo (light green) and an illustrative diffractogram of a β-CD inclusion complex with the same organization pattern (dark green).

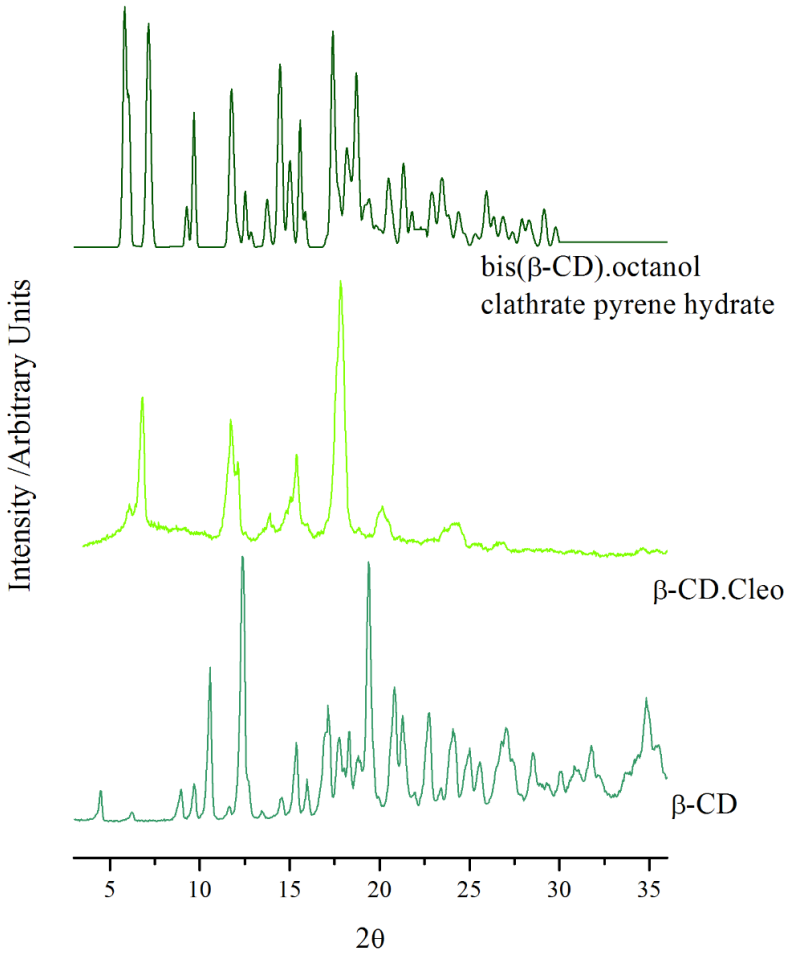

Figure 13 – Powder X-ray diffratogram of β-CD (dark green), β-CD·Cleo (light green) and β-CD channel C2 model, calculated from the atomic coordinates of bis(β-CD)·octanol clathrate pyrene[247] *with the software package Mercury® (darker green).*

Although the obtained pattern appears to be distinct, several similar peaks are present. However, some peaks of β-CD·Cleo are suppressed when compared to channel C2 example. The absence of peaks of the sample in relation to the given example is not problematic and is justified by the fact that the sampling method in absorbance may lead to the suppression of some reflections. The difference in the pattern between the cyclodextrin

and the inclusion complex leads to the conclusion that there has been a restructuring of the host by the presence of a guest in the cavity.

Figure 15 shows the diffractograms of γ-CD (dark blue), γ-CD·Cleo (light blue) and an illustrative diffractogram of a γ-CD inclusion complex with the same organization pattern (medium blue).

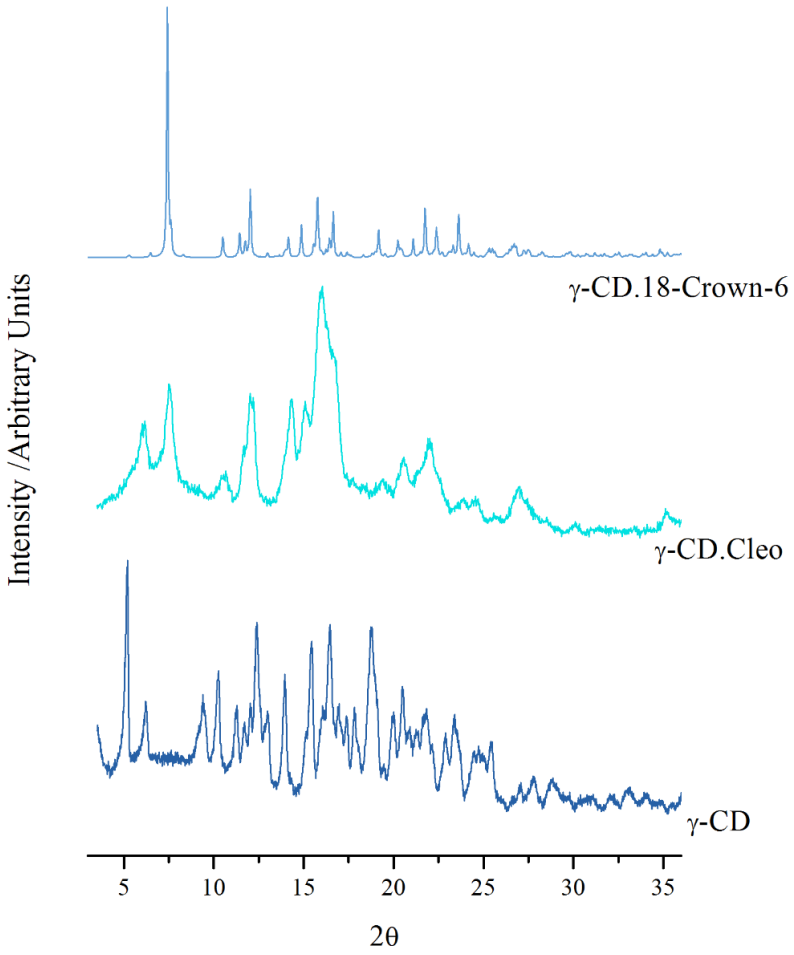

Figure 14 – Experimental PXRD data of γ-CD (dark blue) and γ-CD·Cleo (light blue), and a simulated pattern for a model of γ-CD complex with channel packing. Data was from the single-crystal structure coordinates of γ-CD 18-crown-6 (medium blue)[248], calculated using the software package Mercury®.

γ-CD·Cleo has a pattern distinct from that of pure γ-CD, indicating that the sample is free from contamination with the crystalline host. Furthermore, the pattern has reflections at similar angles of two-theta as those typically observed for channel-packed inclusion complexes (see upper trace in Figure 14), but the reflections are broad and poorly resolved. Nevertheless, it is fair to assume that γ-CD·Cleo units are packing in the form of channels.

3.2. Inclusion Complexes of Gingerols

This work was carried out as a follow-up to a bachelor's degree project, in which the extraction of gingerols from fresh ginger rhizome using acetone maceration was optimized and the effect of cyclodextrins on the antioxidant and anti-inflammatory activities of solutions of gingerols was evaluated *in vitro*[249]. A new extraction of gingerols was carried out and the substance was analyzed for composition and molecular weight. The inclusion complex was then formed with γ-cyclodextrin and it was studied, using infrared vibrational spectroscopy, differential scanning calorimetry, $\{^1H\}$ ^{13}C NMR CP/MAS and powder x-ray diffraction.

Later, gingerols and γ-CD·gingerols were applied in yogurts and the physical characteristics of the fortified yoghurt were evaluated. The β-carotene and α-glucosidase assays were also performed the compound by themselves. The antioxidant activity of the manufactured yogurts was also study by $ABTS^{\cdot+}$ assay.

3.2.1. Extraction and Characterization of the Gingerols Extract

3.2.1.1. Extraction

The gingerols were obtained by maceration with acetone at room temperature, followed by chromatographic purification. Acetone is reported as the most effective solvent for the extraction of 6-gingerol from powdered dry ginger[195]. Note that other compounds of similar structure, 8- and 10-gingerols, share similar physico-chemical properties and therefore they are also present in the final product.

3.2.1.2. Extract Analysis

Gingerols extract was analysed by ^1H NMR, mass spectrometry and infrared vibrational spectroscopy. For a comparison of the pure extract and its γ-CD inclusion complex, analyzes used $\{^1H\}$ ^{13}C NMR (solution phase for the extract and solid-state for the complex) and DSC; the results of these techniques will be presented in the corresponding subchapters, below.

3.2.1.2.1. ^{1}H NMR in Solution

The ^{1}H NMR spectrum of the extract, dissolved in $CDCl_3$, is presented in Figure 15. Data was compared with that of the sample previously prepared by the bachelor student Bruna. The existing sample was denominated Fraction 1, and the gingerol extract herein prepared was denominated Fraction 2. Results are summarized in Table 24.

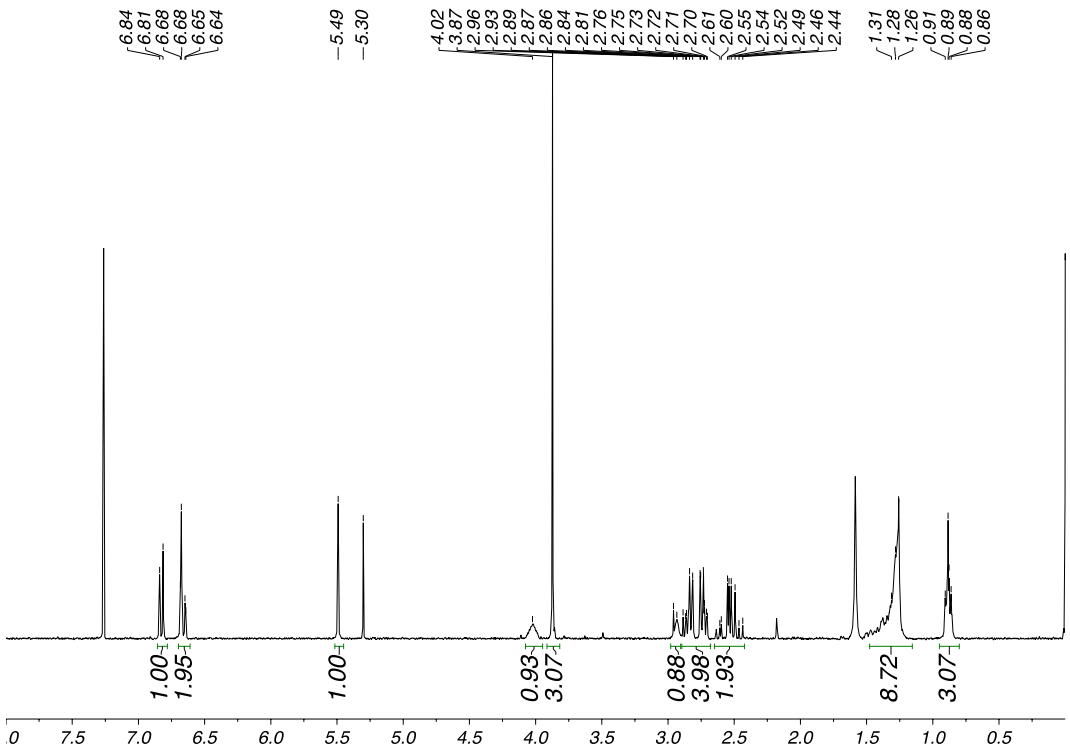

Figure 15 – ^{1}H NMR analysis of gingerols sample obtained by acetone extraction from ginger rhizome, also identified as Fraction 2. The peak at 5.30 ppm is referent to the used solvent.

Figure 16 shows the structure of 6-gingerol, the major compound in both samples. Table 24 below shows the spectral data obtained for both samples as well as their integration. Each hydrogen was further identified with the literature[221]. The data obtained of ^{1}H NMR for Fraction 1 is presented in Appendix C.

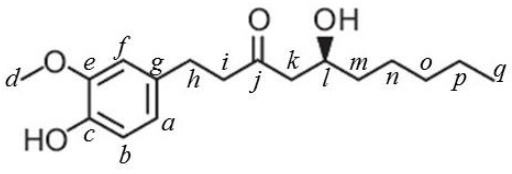

Figure 16 – Structure of 6-gingerol.

Table 24 – Spectral data of the ^{1}H NMR of 6-gingerol, Fraction 1 (previously obtained sample) and Fraction 2 (sample obtained in the present work).

	Literature[221]		**Fraction 1**		**Fraction 2**	
	δ (ppm)	**Integration**	**δ (ppm)**	**Integration**	**δ (ppm)**	**Integration**
***q*-CH_3**	0.86	3.0341	0.86-0.91	3.13	0.86-0.91	3.07
p*-(CH_2)*n*-*m	1.25-1.59	8.4582	1.28-1.39	8.78	1.26-1.31	8.72
k*-CH_2-*j	2.44-2.59	2.0309	2.44-2.61	1.92	2.44-2.61	1.93
i*-CH_2-*h	2.70-2.76	1.9902	2.70-2.87	4.01	2.70-2.89	3.98
g*-CH_2-*a	2.81-2.87	1.9823				
H*l*	2.93	0.9519	2.94	0.65	2.93-2.96	0.88
***d*-CH_3**	3.87	2.9818	3.87	3.05	3.87	3.07
***l*-OH**	4.05	1.000	4.02-4.04	1.03	4.02	0.93
***c*-OH**	5.50	0.9689	5.50	0.97	5.49	1.00
H-*a*, H-*b*	6.64-6.71	1.9703	6.64-6.68	2.01	6.64-6.68	1.95
H-*f*	6.78-6.84	0.9798	6.81-6.84	1.00	6.81-6.84	1.00

Integrations match the expected values for all protons with the exception of *p*-(CH_2)*n*-*m* which is slightly higher, denoting the contribution of 8- and 10-gingerols, that have more C*H*$_2$ protons.

3.2.1.2.2. Mass Spectrometry

The extract was analyzed by TOF MS ES$^+$, results presented in Figure 17.

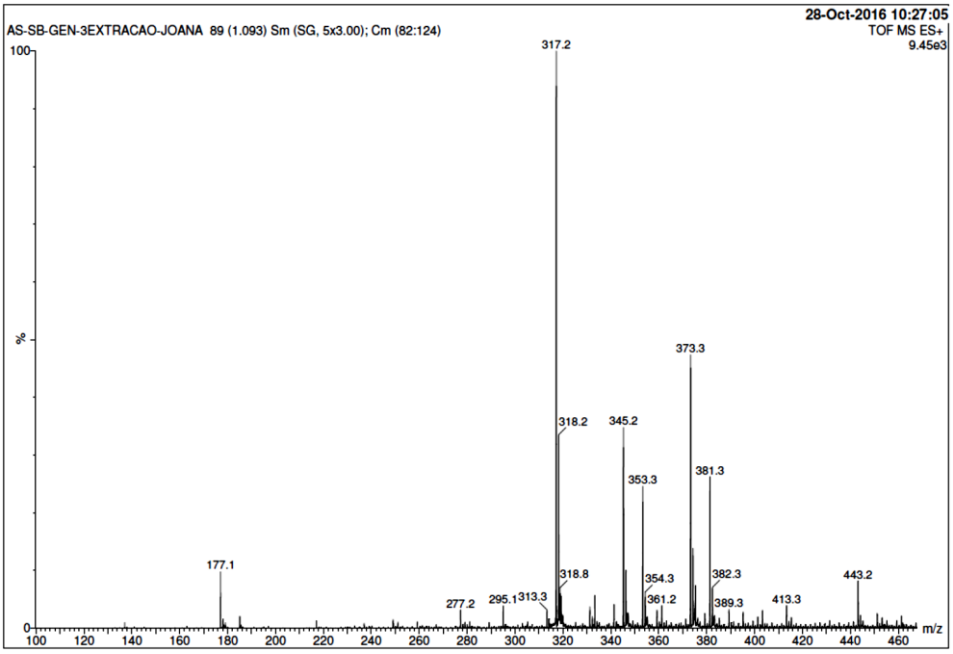

Figure 17 – Mass spectrum obtained for ginger rhizome extract in acetone.

The most intense peaks correspond to the monocations formed by gingerols with an adsorbed sodium. By subtraction of the sodium mass (Mr (Na^+) = 22), it is known that *m/z* = 317.2 corresponds to 6-gingerol, *m/z* = 345.2 corresponds to 8-gingerol and *m/z* = 373.3 to 10-gingerol. Relative to the percentages obtained in the graph, where 100% corresponds to 6-gingerol and the percentages of 8-gingerol and 10-gingerol are 35% and 47.5% respectively. This means that the extract has 54.05% of 6-gingerol, 19.45% of 8-gingerol and 26.5 % of 10-gingerol, which implicates a molecular mass of 314.71 g/mol, as shown in Table 25. The sample will be called "gingerols" in the remaining subsections

Table 25 – Ginger rhizome extract composition.

m/z	**Compound**	**Molecular Weight (g/mol)**	**Quantity (%)**	
317.2	**6-gingerol**	294.38	54.05	159.11
345.2	**8-gingerol**	322.44	19.45	62.71
373.3	**10-gingerol**	350.49	26.50	92.88
		Total Molecular Weight (g/mol)		**314.71**

3.2.1.2.3. Fourier Transform Infrared Vibrational Spectroscopy Analysis (FT-IR)

The spectra of the gingerols and γ-CD·gingerols are presented in the Figure 18. Gingerols are identified in orange and inclusion complex in blue.

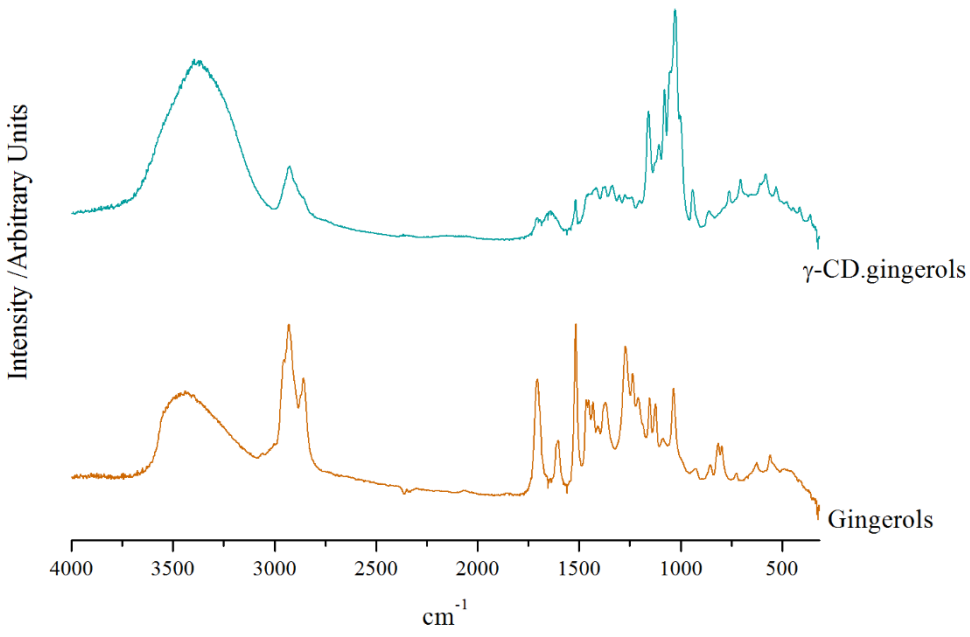

Figure 18 – FT-IR spectra of gingerol and γ-CD·gingerols.

Between 2800 and 3000 cm^{-1}, the presence of several signals in the gingerols interferogram is observed, while in the inclusion complex are reflected in a signal of lower intensity and number of peaks – Table 26.

Table 26 – Selected gingerol bands in the spectra of gingerols and γ-CD·gingerols.

Gingerols	**γ-CD·Gingerols**	**6-Gingerol, description**[250]
1707	1707	1727, ν(C=O)
1602	—	1652, ν(C=O)
1515	1516	1517, ν(C=C)

The corresponding bands were identified taking into account the contribution of γ-cyclodextrin, so that the area of great contribution of the CD was discarded. With the encapsulation, the intensity of the same peaks diminished, as well as some small deviation, in relation to the gingerols sample.

3.2.2. γ-CD·Gingerols Inclusion Complex

In a similar fashion to the inclusion complexes with *Cistus ladanifer* essential oil, inclusion of gingerols was performed by co-precipitation, working close to the saturation of γ-CD to favour the precipitation of the complex. The complex was characterized by Fourier transform infrared spectroscopy (FTIR), {^{1}H} ^{13}C CP-MAS NMR, differential scanning calorimetry (DSC) and powder X-ray diffraction.

3.2.3. {^{1}H} ^{13}C CP-MAS NMR

Gingerols and γ-CD·gingerols inclusion complex were analysed by {^{1}H} ^{13}C CP-MAS NMR. Gingerols analysed on its own allowed later to correlate with the data obtained for the inclusion complex sample. It is only stated that the gingerols sample was analysed in the liquid phase in deuterated chloroform and the inclusion complex was in the solid state. Figure 19 presents the {^{1}H} ^{13}C NMR spectra for gingerols (orange) and for both γ-CD (dark blue) and γ-CD·gingerols (light blue).

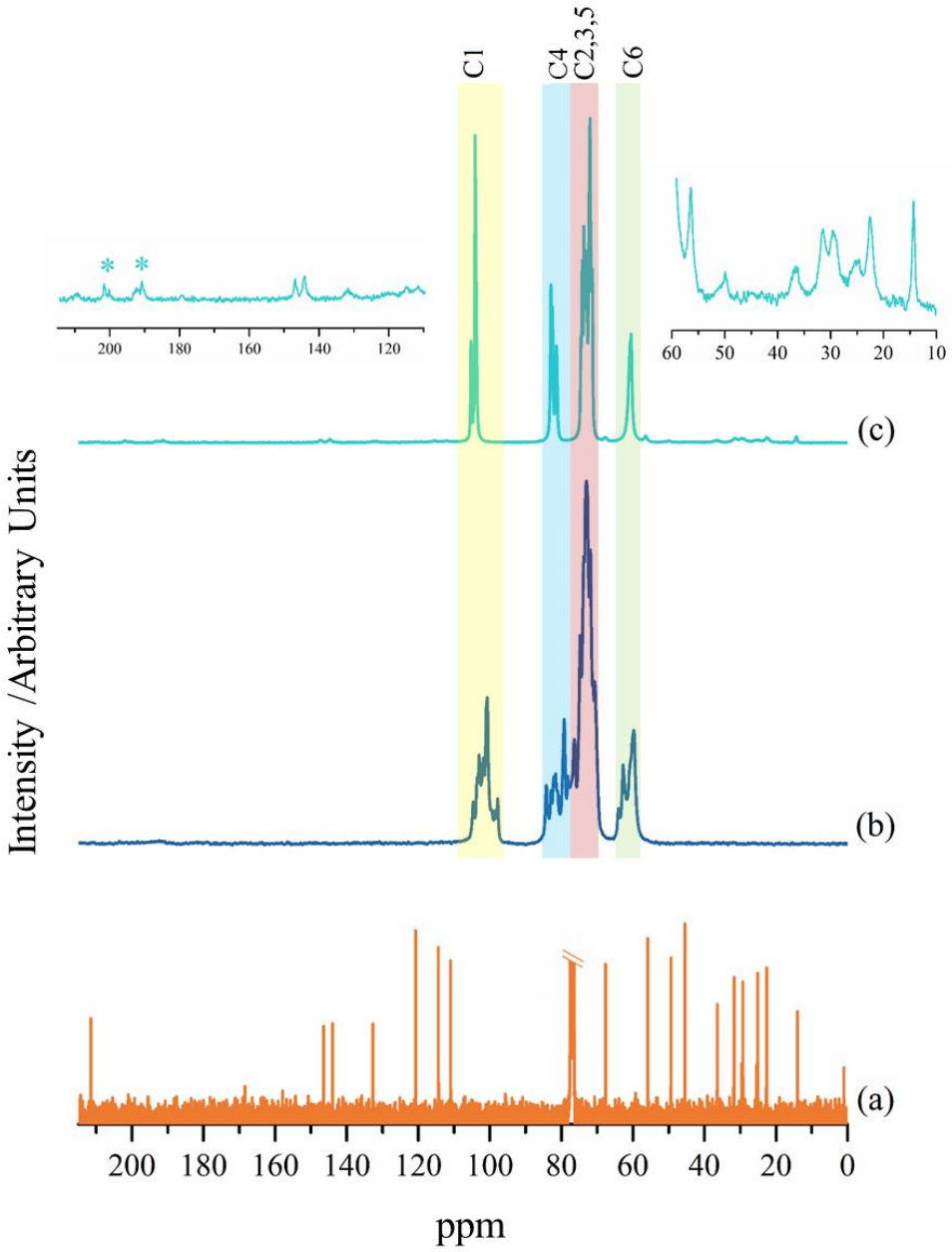

Figure 19 – {^{1}H} ^{13}C CP/MAS NMR analysis of gingerols (a), of γ-CD (b) and γ-CD·gingerol (c). γ-CD and γ-CD·gingerol have assignments according to the literature[241]*. In γ-CD·gingerol, there are sppining sidebands from γ-CD influence (*)*

6-Gingerol peaks were identified by comparison with reported data 6-gingerol, as listed in Table 27.

Table 27 – Attribution of gingerol carbon resonances.

	Literature[221]	Gingerols	γ-CD·Gingerol
C***q***	13.9	14.0	13.9
C***p***	22.5	22.6	22.5
C***n***	24.9	25.1	25.0
C***o***	29.1	29.3	29.5
C***h***	31.6	31.7	31.7
C***m***	36.3	36.4	36.7
C***i***	45.3	45.4	50.0
C***k***	49.3	49.3	
C***l***		55.9	56.7
C***d***		67.7	67.5
C***f***	110.9	111.0	111.7
C***b***	114.4	114.4	115.4
C***a***	120.6	120.7	
C***g***	132.5	132.6	132.5
C***c***	143.9	144.0	144.6
C***e***	146.4	146.4	147.5
C***j***	211.3	211.5	210.0

3.2.4. Differential Scanning Calorimetry (DSC)

Gingerol and inclusion complex were analysed by differential scanning calorimetry (DSC). Results are presented in the Figures 20 and 21.

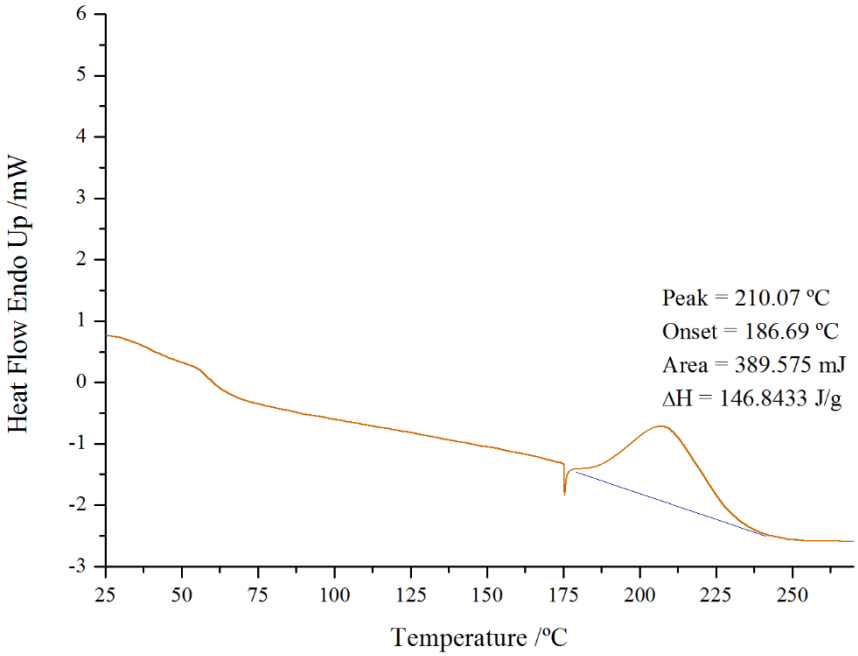

Figure 20 – Differential scanning calorimetry analysis of gingerol.

The sample containing gingerols presents a phase change with the onset at 186.69 ºC and peaking at 210.07 ºC. The area is 389.575 mJ, with a ΔH of 146.8433 mJ/mg.

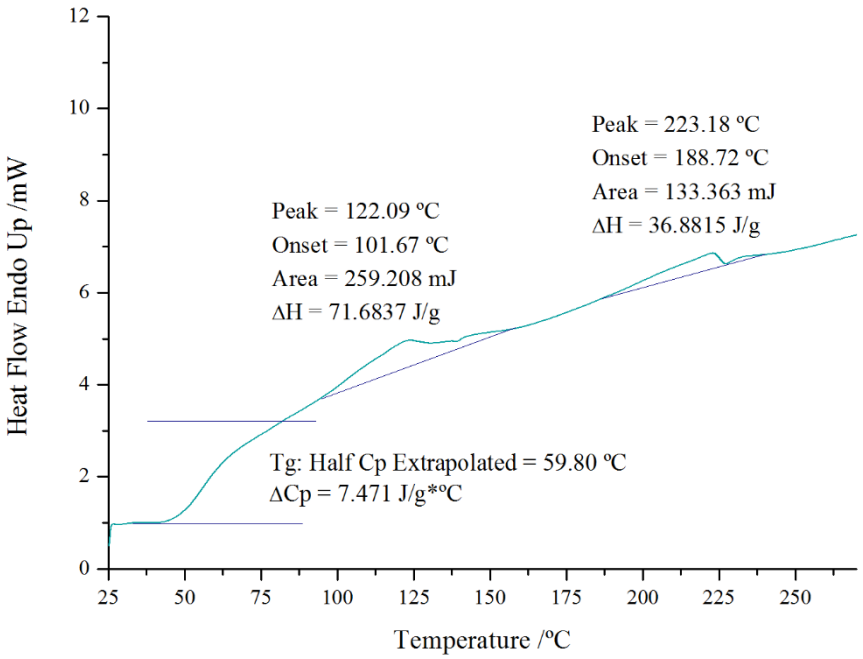

Figure 21 – Differential scanning calorimetry analysis of γ-CD·gingerol.

For the inclusion complex, three events can be observed in the DSC trace. The first two events, associated with the loss of hydration water molecules are observed with centres at 59.80 ºC, and 122.09 ºC. The loss of water molecules in two different steps is typical for CD inclusion complexes[251] as some molecules are less tightly bound (usually these are clustered in spaces between the cyclodextrins), while other molecules, in stronger interaction with host or even guest molecules, are more tightly bound. Following, there is a third event centred at 233.18 ºC, with onset at 188.72 ºC and area of 133.363 mJ (ΔH of 36.8815 mJ/mg). This last transition can be associated with the endothermic peak of gingerols. Although it peaks at a slightly different temperature, possibly because of gingerols encapsulated in cyclodextrin, the onset occurs at the same temperature.

3.2.5. Powder X-Ray Diffractograms of Gingerols Extract Inclusion Complex

The inclusion complex with gingerols as host was analyzed by powder x-ray diffraction. After a scan that took 10 minutes, the sample was analyzed overnight, with differentiation in the slits used, in order to increase the resolution – Figure 22.

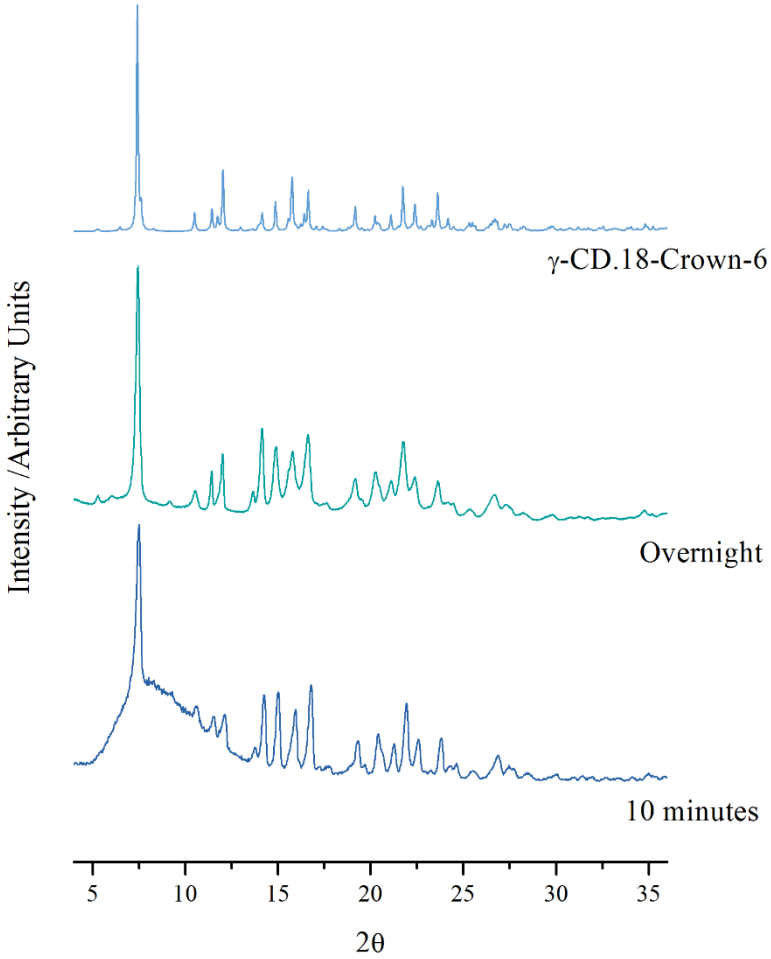

Figure 22 – Powder diffractograms of γ-CD.gingerols (bottom two) under different conditions and a simulated pattern for a model of γ-cyclodextrin complex with channel packing. Data was from the single-crystal structure coordinates of γ-CD 18-crown-6 (medium blue)[248], calculated using the software package Mercury®.

The trace collected in 10 minutes presents very low resolution, so the sample was re-collected overnight, for higher signal intensity, and using narrow slits for noise minimization. The new diffractogram presents good resolution and adequate intensities for data modelling. The diffraction pattern presented in both data corresponds to the standard already defined for structures with cyclodextrins with guest organized in channel, and it can be postulated that the structure of γ-CD·gingerols is in channel.

Thus, through software, a Pawley's fit was applied, using the atomic coordinates of known structure, γ-CD·18-crown-6-ether, it was possible to obtain the γ-CD·gingerols cell parameters – Figure 23.

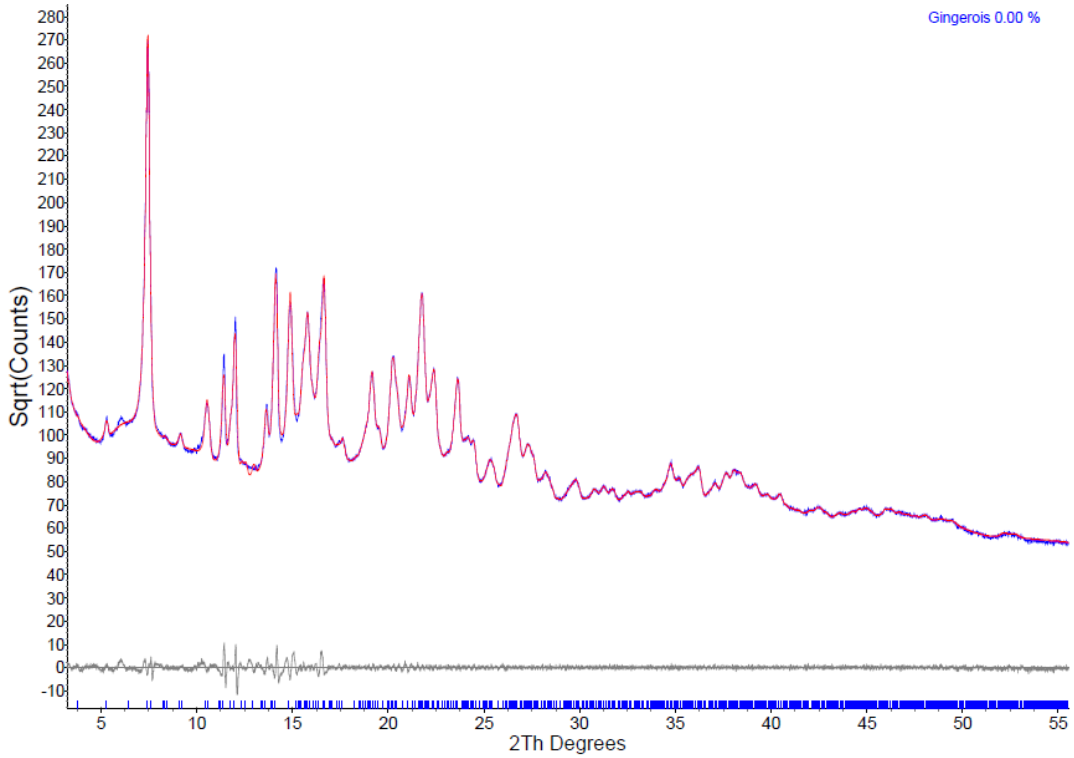

Figure 23 – Pawley's fit on powder diffractograms of γ-CD.gingerols.

In the noise line present in the upper figure, it is still possible to observe at low angles the presence of impurities. Nevertheless, it was possible to establish the parameters of the cell. The organization of γ-CD·gingerols cell is tetragonal, with a = b = 23.886(3) Å and c = 23.356(3) Å and α = β = γ = 90 º.

3.3. Yogurt with Gingerol

By reproducing the homemade production of yogurt, yogurts fortified with pure or encapsulated gingerols were made in the laboratory using a thermostated water bath (details in the subsection 2.2.8). Figure 24 shows the resulting products. Vials identified from 1 to 3 are plain yogurts, while the yogurts 4 to 6 and 7 to 9 correspond to yogurts fortified with gingerols and γ-CD·gingerols, respectively. Samples were made in triplicate for each fortification and masses were adjusted to have a fortification of 1% of gingerols or its equivalents (in the complexes) in each yogurt. Only sample 6 was prepared in a smaller amount, because the gingerols mass was not sufficient.

Figure 24 – Photograph of yogurt produced in the laboratory – from left to right, the first three yogurts were not supplemented, the next three were fortified with gingerol, and to the last three was added γ-CD·gingerols.

3.3.1. pH variation

The pH of the various samples was measured after one two and weeks of storage at 4 ºC. Table 28 shows the pH values in two weeks.

Table 28 – pH measurements on samples of simple and fortified yogurt with gingerois and γ-CD·gingerols.

Sample	Week 1		Week 2		Week 3		Week 4	
	pH	***Odour***	***pH***	***Odour***	***pH***	***Odour***	***pH***	***Odour***
Simples Yogurt	3.71 ± 0.06	—	3.73 ± 0.02	—	3.62 ± 0.04	+	3.59 ± 0.06	++
Gingerols Yogurt	3.72 ± 0.07	—	3.78 ± 0.06	—	3.65 ± 0.06	—	3.60 ± 0.04	—
Complex Yogurt	3.65 ± 0.06	—	3.71 ± 0.01	—	3.59 ± 0.04	—	3.56 ± 0.07	—

The pH of the yogurts was measured during 4 weeks, with changes in values being found in the tenths of a unit. Regarding the odour, from the third week, there were organoleptic changes on the simple yogurts, being this still more active in the fourth week.

3.3.2. Colorimetric Assay

The equipment used allows to know the parameters L^*, a^* and b^*. Figure 25 demonstrates how color composition can occur with the variation of these three parameters.

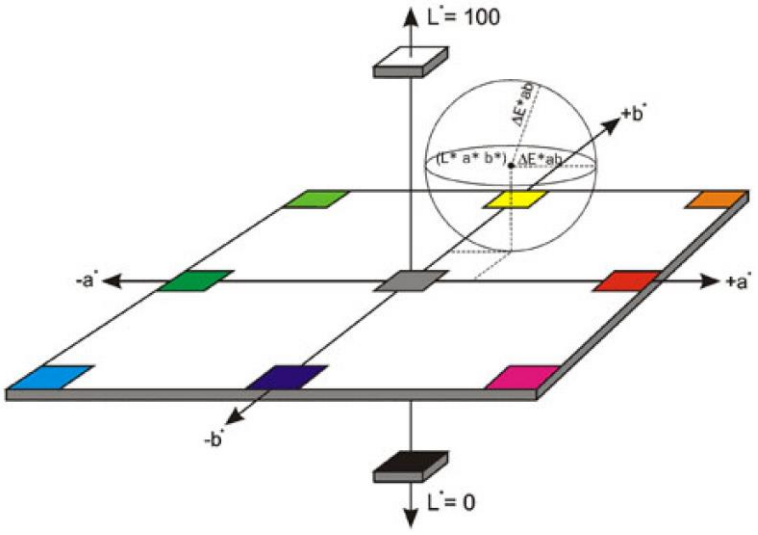

Figure 25 – Graphical interpretation of the parameters analysed: L^* – black/white, $+a^*/-a^*$ – red/green, $+b^*/-b^*$ = blue/yellow[252].

The nine samples of simple and fortified yogurt were evaluated for color to see if there was a difference between normal and fortified yogurt – data in Table 29.

Table 29 – Colour parameters obtained for all samples and media (ȳ) obtained for the several parameters.

Yogurt	***L****	***a****	***b****	**ȳ (*L**)**	**ȳ (*a**)**	**ȳ (*b**)**
1 *(n = 5)*	88.980	– 1.408	3.500			
2 *(n = 5)*	92.418	– 1.548	5.302	87.986 ± 5.003	– 1.317 ± 0.286	4.086 ± 1.053
3 *(n = 5)*	82.561	– 0.997	3.456			
4 *(n = 10)*	90.587	– 1.830	6.524			
5 *(n = 10)*	91.044	– 2.048	7.035	89.076 ± 3.020	– 1.874± 0.155	6.646 ± 0.344
6 *(n = 10)*	85.598	– 1.746	6.380			
7 *(n = 5)*	83.964	– 1.372	3.678			
8 *(n = 5)*	85.240	– 1.520	6.536	87.615 ± 5.258	– 1.620 ± 0.310	5.968 ± 2.066
9 *(n = 5)*	93.642	– 1.968	7.692			

With the values of ȳ (L^*), ȳ (a^*) and ȳ (b^*), it is possible to calculate the associated error (ΔE) between the reference sample, i.e., plain yogurt, and the innovative samples. Thus, Equation 4 was applied, in which ΔL^*, Δa^* and Δb^* is the difference in that parameter between reference sample and the gingerol and γ-CD·gingerols yogurt[253].

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad - \textit{Equation 4}$$

Thus, for the gingerols yogurt, a ΔE = 2.84 was obtained, whereas for the γ-CD·gingerols yogurt, the ΔE decreased to 1.94. Note that the standard list defines that a ΔE between 0 and 1, the observer does not notice the difference; between 1 and 2, experienced observer can notice the difference; between 2 and 3.5, unexperienced and experienced observers notice the difference; between 3.5 and 5, it is noticed clear difference in colour; and, above 5, observers notice two different colours[252].

According to that, it would be possible to an unexperienced observer notice the difference between simple yogurt and gingerols yogurt, and it would be possible to an experienced observer can notice the difference between simple yogurt and γ-CD·gingerols yogurt.

The information obtained complements the observation made with the naked eye in the sample of gingerols yogurt, as it is possible to identify small droplets in yogurt. Figure 26 allows to observe the yellow droplets of gingerols in the yogurt.

Figure 26 – Gingerol droples in yogurt.

The presence of these droplets may influence the larger difference between plain yogurt and yogurt with gingerols, since during the collection of data the sample was not uniform. In addition, the equipment is specific for solid samples, which alone causes three errors. Firstly, the surface of the yogurt is not smooth, in which, during the data collection, the luminosity to which the sample is submitted differs, that is, the parameter L^* has greater variation. Then the fact that the sensor is at a variable distance from the sample, since the sample can not touch the sensor, with the danger of spoiling it. Finally, and also related to the distance, the greater distances between the equipment and the sample there is a greater influence of the external luminosity. In order to reduce these sources of error, 5 or 10 replicates were collected for each sample.

It is possible, nevertheless, to associate the obtained parameters with the well-known RGB coordinates and to transform these into (x, y) coordinates, in order to apply them on a (x, y) Chromaticity Diagram. The conversion was obtained using two websites: from (L^*, a^*, b^*) to (RGB)[254], and in (x, y) coordinates[255]. The coordinates for each sample in the different colour models are presented in Table 30 and the (x, y) coordinates were applied in a CIE 1931 Chromaticity Diagram, defined in a (x, y) referential, presented in Figure 27.

Table 30 – Table of coordinates conversion.

	L^*, a^*, b^*	R, G, B	Y, x, y
Simple Yogurt	87.986, – 1.317, 4.086	221, 221, 213	72.572, 0.31813, 0.33796
Gingerols Yogurt	89.076, – 1.874, 6.646	224, 225, 211	74.964, 0.32111, 0.34454
γ-CD·gingerols Yogurt	87.615, – 1.620, 5.968	221, 220, 208	71.860, 0.32191, 0.34252

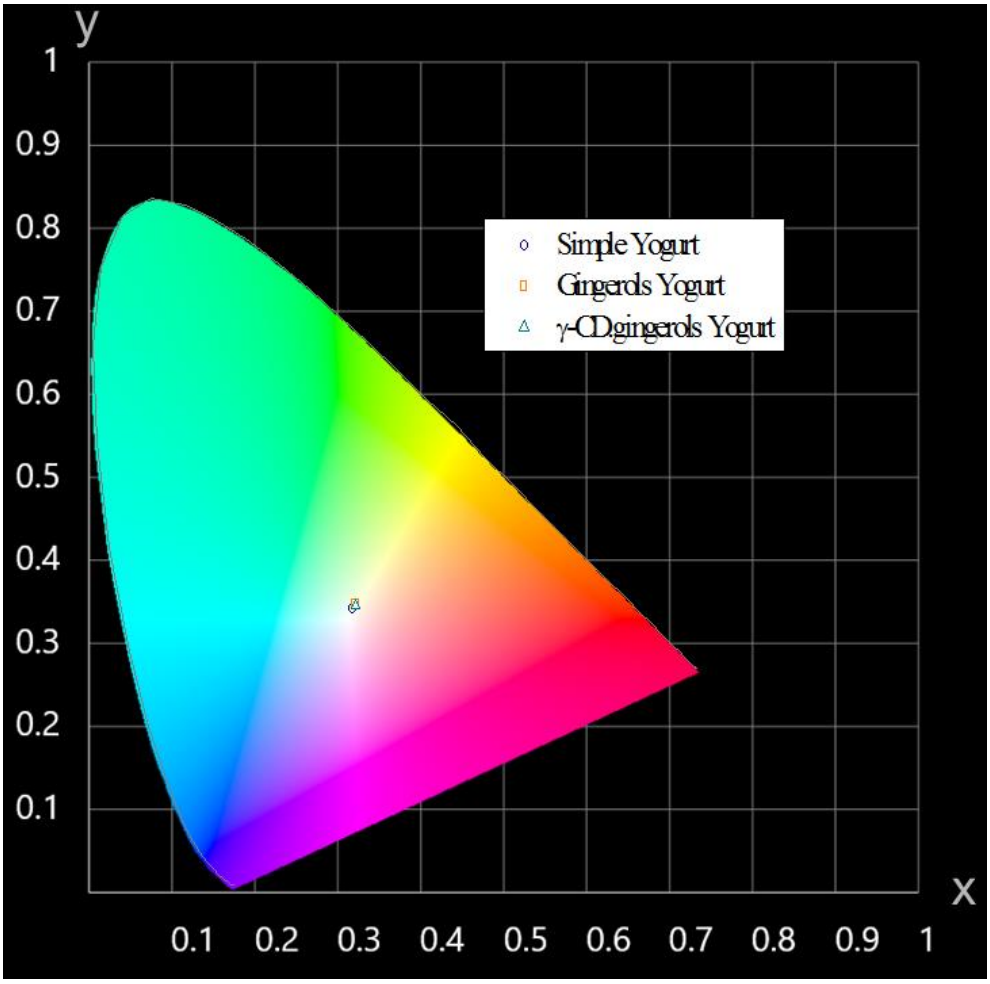

Figure 27 – Chromaticity diagram with the representation of simple and fortified yogurt samples.

3.4. Gingerols Biochemical Assays

The antioxidant activity of gingerols, both pure and in the presence of CD, was evaluated in previous work by Bruna Pereira, using the by $ABTS^{\cdot+}$, 5-LOX inhibition and $NO^{\cdot}$ scavenging assays[249]. Gingerols were shown to have a good antiradical activity towards $ABTS^{\cdot+}$, that was quite similar to that of the reference, Trolox. Gingerols also inhibited 5-LOX, but their $NO^{\cdot}$ scavenging ability was poor. Table 31 summarises the obtained results.

Table 31 – Biochemical data from previous work with gingerols in the presence and absence of γ-CD[249].

	Biochemical Assay		
	$ABTS^{\cdot+}$	**5-LOX**	**$NO^{\cdot}$(% of inhibition)**
Standard Solution	*Trolox* EC_{50} = 7,99±0,99 μM (2,00±0,25 μg/mL)	*Ascorbic acid* EC_{50} = 234 ± 21 μM (0.0412 ± 3.7 μg/mL)	*Ascorbic acid (c = 1 mM)* 50.3 ± 1.9 %
γ-CD	—	42 % of inhibition (1000 μM)	11.3 ± 1.5%
Gingerols	EC_{50} = 9.13 ± 1.03 μM (2.83 ± 0.32 μg/mL)	EC_{50} = 695 ± 47 μM (0.216 ± 0.015 mg/mL)	17.5 ± 3.2 %
γ-CD·gingerols	EC_{50} = 8.80 ± 0.81 μM (2.73 ± 0.25 μg/mL)	EC_{50} = 629 ± 101 μM (0.195 ± 0.031 mg/mL)	25.3 ± 1.2 %

3.4.1. β-Carotene Assay

The antioxidant activity of gingerols, γ-CD and γ-CD·gingerols was further evaluated by the β-carotene assay, which uses this substrate in an emulsion with linoleic acid. This study allowed to evaluate the antioxidant activity of gingerols and γ-CD·gingerols, since oxidation of β-carotene by linoleic acid occurs in the absence of antioxidants.

Results are represented in Figure 28. Note that γ-CD showed no antioxidant activity and therefore its percent inhibition graph is found in the Appendix E. The reference substance, BHA, used to evaluate the efficiency of the formulated emulsion thus verify the validity of this assay, is given in the Appendix D.

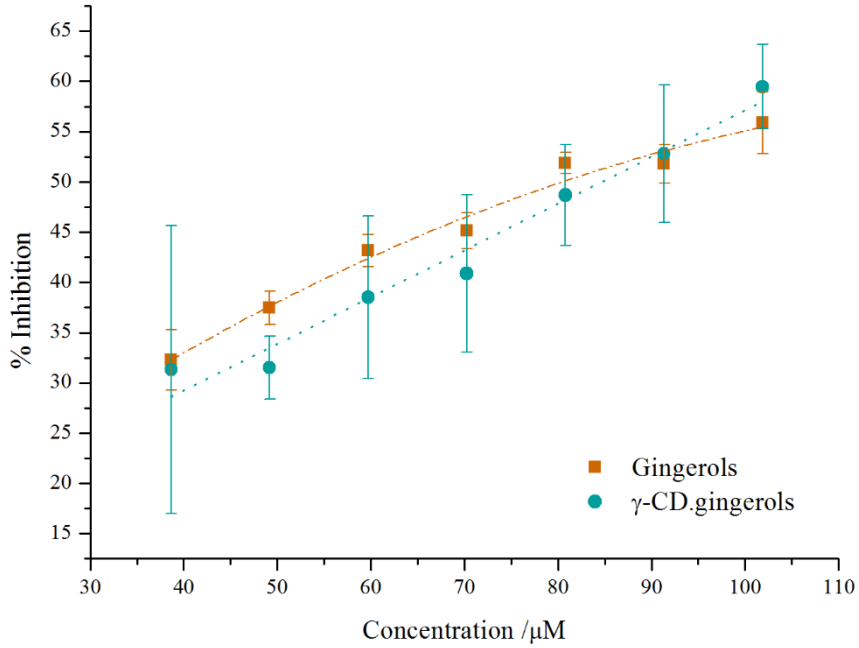

Figure 28 – Inhibition of β-carotene bleaching by gingerols and γ-CD·gingerols at different concentration. Data is represented as mean ± sd of three independent assays. Dashed lines represent the bleaching behaviour of the samples.

The EC_{50} values for the two samples are very similar: EC_{50} = 83.3 ± 4.7 μM for gingerols and EC_{50} = 85.1 ± 7.1 μM for γ-CD·gingerols. EC_{50} values are not statistically significant, which means that the activity of the inclusion complex whose host is gingerols is equal to that of gingerols. Thus, cyclodextrin encapsulation does not alter the activity of this host, as it was observed in γ-CD's antioxidant activity using this assay.

3.4.2. α-Glucosidase Assay

The α-glucosidase inhibition assay was first tested using a high concentration of gingerols (0.325 mg/mL, equivalent to 1.03 mM). At this concentration, gingerols displayed about 4.4 % inhibition, meaning that they are not good inhibitors of the α-glucosidase enzyme; no further studies thus were pursued regarding this system.

3.4.3. $ABTS^{\cdot+}$ Assay in Yogurt Samples

Further testing of antioxidant activity was performed by altering the matrix in which the compounds were included. In this assay, the dark green radical $ABTS^{\cdot+}$ is generated by oxidation of ABTS with potassium persulfate ($K_2S_2O_8$). The $ABTS^{\cdot+}$ solution suffers decolorization in the presence of hydrogen-donating antioxidants.

Test samples comprised a hydroethanolic extract of the yogurts. The use of 1 mL of ethanol had 3 objectives: first, since gingerols are hydrophobic, it assist in their incorporation into the extract; second, it increases the extraction power of the compounds under study; and, finally, it reproduce, as much as possible, the tested conditions used in the $ABTS^{\cdot+}$ assay, made with ethanol:water 1:1 solutions. The design of the assay comprised defining the activity of the compounds in ascorbic acid equivalent, whereby the obtained graphic is in absorbance as a function of the concentration of ascorbic acid – Figure 30.

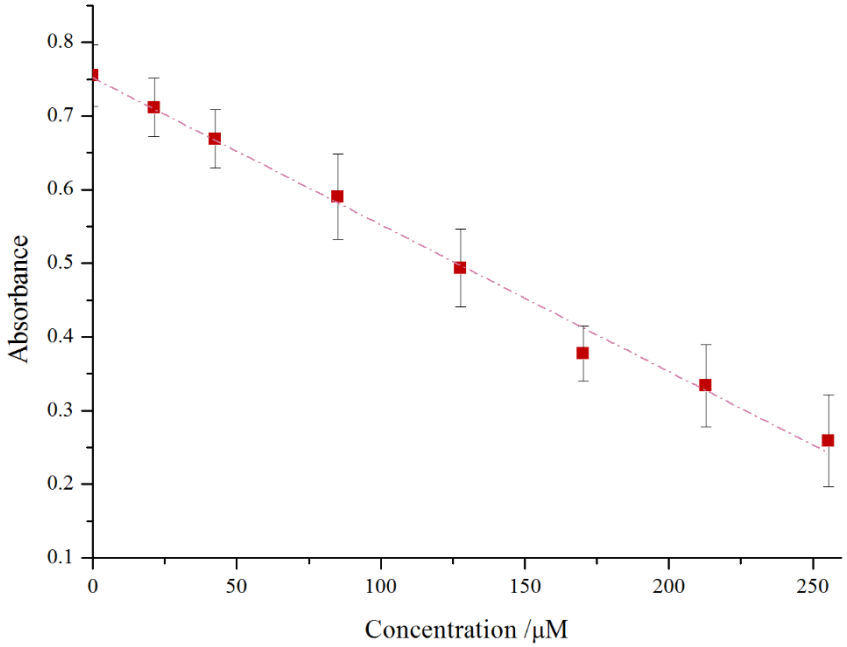

Figure 29 – Ascorbic acid absorbance as a function of acid ascorbic concentration. Data is represented as mean ± sd of three independent assays. Dashed lines represent the expected behaviour ascorbic acid in other concentrations.

Table 32 – Test summary table: it presents the selected dilution factors, concentration of fortified compound in the dilution and the concentration related to ascorbic acid, extrapolated from Figure 29.

Yogurt	Dilution factor	Concentration of gingerols or γ-CD·gingerols /µM	Concentration from Figure 29 /µM
1	4	—	106.7
2		—	101.4
3		—	101.7
4	40	302.50	211.9
5		307.22	166.4
6		330.32	148.2
7	28	105.00	90.9
8		103.66	135.4
9		103.24	117.4

Taking into account the dilutions and comparing with ascorbic acid, it can be defined that simple yogurt has a similar conduct to 0.826 ± 0.023 mM of ascorbic acid and, in fortified yogurts, gingerols yogurt has 14.041 ± 2.624 mM of ascorbic acid and γ-CD·gingerols yogurt has 6.416 ± 1.254 mM of ascorbic acid.

As can be observed, gingerols-fortified yogurt showed the highest antioxidant capacity as compared to the remaining formulations. γ-CD·gingerols fortified yogurt also showed higher antioxidant capacity than that of the plain yogurt, thus indicating that the introduction of γ-CD·gingerols in complex matrix allow to improve their functionality.

Note still that the antioxidant activity in γ-CD·gingerols yogurts was about half that of gingerols-fortified yogurt, even though the complex allowed to increase the solubility of these compounds. As previous $ABTS^{\cdot+}$assay with gingerols and γ-CD·gingerols showed similar $ABTS^{\cdot+}$ scavenging abilities[249], the present results thus suggest that the antioxidant ability of γ-CD·gingerols in a complex milk matrix might be partially impaired in comparison to that observed in a simple solution.

4. Conclusion

The main objective of the work, that is, to obtain and characterize inclusion complexes with multicomponent guests of plant origin, was successfully achieved.

The composition of the guests was studied prior to inclusion. *Cistus ladanifer* essential oil (Cleo) was analysed by GC-MS, while gingerols extract was evaluated by MS and ^{1}H NMR. Following inclusion, the process of preferential encapsulation was demonstrated for β-CD·Cleo and γ-CD·Cleo. Indeed, GC-MS analysis of the included material revealed a different composition than that of the guest, only a few of the components being included. The composition also varied according to the host employed, which was expected due to the slight difference in cavity size.

These inclusion complexes would be applied in cosmetic products, specifically for young people with acne. As described in the introduction, the essential oil has antimicrobial properties. It would then be necessary to investigate whether encapsulation allowed to maintain this biological property. Using the inclusion complex, it would be possible to obtain a uniform and non-cloudy topical gel, a dermocosmetic product with the desired biological and visual properties.

γ-CD·gingerols form a microcrystalline inclusion complex with a channel structural organization, as proved with PXRD. Fortified yogurts were made by the addition of gingerols and γ-CD·gingerols. The colour of the product changed slightly in regard to that of plain yoghurts, but this difference cannot be perceived by the average observer/consumer, only rather by an experienced observer. pH monitoring for two weeks did not show any changes, either for plain or fortified yoghurt (observation for a third week is going to establish if the gingerols are able to increase the shelf-life of the yoghurt). It is also noted that the dissolution of the gingerols into the milk matrix was not complete, so the formation of the inclusion complexes increased the solubility.

Biochemical tests have also been carried out. The β-carotene assay showed no difference in the antioxidant activity of gingerols, either alone or in the form of inclusion complex. This result was corroborated by the absence of activity of γ-CD. The $ABTS^{\cdot+}$ assay performed on yogurt extract, showed that gingerols retain their antioxidant activity. In this case, the differences between the action of gingerols and γ-CD·gingerols are more noticeable, in which the addition of 1% of compound is more effective in the case of gingerols.

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Appendices

Appendix A – *Cistus ladanifer* GC-MS Analyse (graphic figure)

Figure 30 shows the GC-MS graph for *Cistus ladanifer* essential oil – 10 µL of essential oil in 1 mL of chloroform.

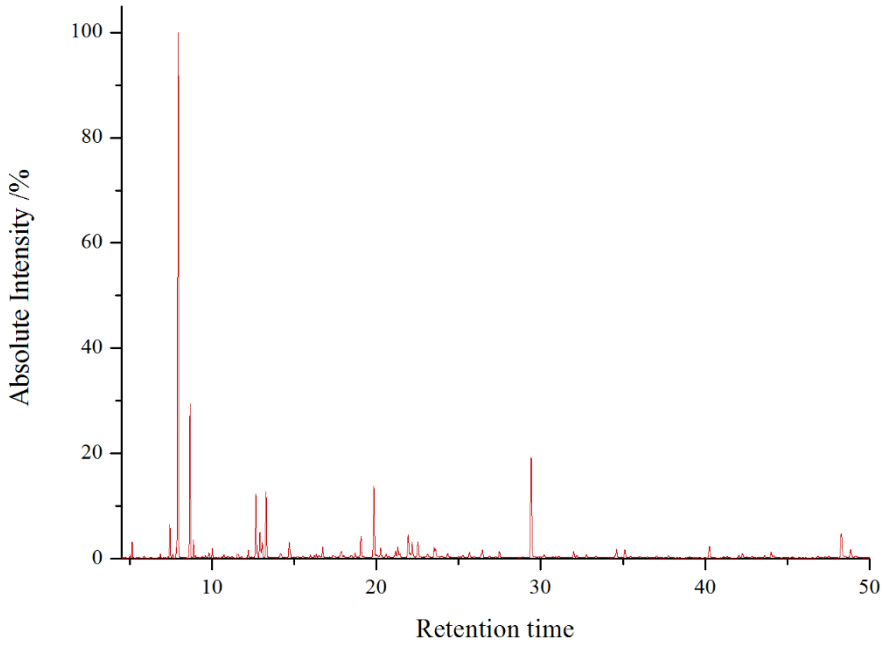

Figure 30 – Graph of GC-MS obtained for the sample of Cistus ladanifer essential oil.

Appendix B – *Cistus ladanifer* GC-MS Analyse (table)

Using the GCMS Postrun Analysis program, the following assignment was made – Table 33. The correspondence was confirmed by the bibliography. In some cases, only the molecular mass is presented, as where there was no concordance or similarity of less than 85% but which had the same molecular weight.

The essential oil was defined as 143.685 g/mol molecular weight, with 5.69% unknown.

Table 33 – Composition of Cistus ladanifer essential oil.

Ret. Time	Start TM	End TM	% Area	% Height	Compound Name	Formula	Mol Weight	Bibliographic Similarity
4.493	4.467	4.520	0.02	0.05	—			
5.013	4.955	5.052	0.12	0.21	—			
5.145	5.093	5.190	0.59	1.04	—	C_9H_{16}	124	
5.861	5.810	5.917	0.10	0.11	—			
6.866	6.820	6.910	0.16	0.24	—	$C_{10}H_{18}$	138	
7.339	7.302	7.370	0.04	0.06	(L)-Linalool	$C_{10}H_{18}O$	154	143,147,148
7.439	7.377	7.508	1.67	2.25	Tricyclene	$C_{10}H_{16}$	136	138,141,144,146,256
7.624	7.567	7.672	0.17	0.25	α-Thujene	$C_{10}H_{16}$	136	141,143,146,149,256
7.825	7.770	7.858	0.49	0.68	—	$C_8H_{16}O$	128	
7.947	7.860	8.042	30.12	35.44	α-Pinene	$C_{10}H_{16}$	136	131,138,141,143–149,256
8.667	8.538	8.757	8.58	10.41	Camphene	$C_{10}H_{16}$	136	131,138,141,143–147,149,256
8.872	8.807	8.942	0.94	1.18	Verbenene	$C_{10}H_{14}$	134	146,256
8.997	8.953	9.042	0.08	0.14	—			
9.414	9.282	9.470	0.08	0.05	—			

Ret. Time	Start TM	End TM	% Area	% Height	Compound Name	Formula	Mol Weight	Bibliographic Similarity
9.602	9.550	9.665	0.11	0.14	—	$C_8H_{14}O$	126	
9.820	9.763	9.918	0.21	0.31	Sabinene	$C_{10}H_{16}$	136	[138,141,143,145,146,256]
10.028	9.953	10.125	0.55	0.63	β-Pinene	$C_{10}H_{16}$	136	[141,143–145]
10.607	10.562	10.637	0.04	0.06	6-Methyl-5-hepten-2-one	$C_8H_{14}O$	126	
10.717	10.662	10.818	0.22	0.19	Verbanol	$C_{10}H_{16}O$	152	[130,149]
10.974	10.905	11.033	0.10	0.11	—	$C_{10}H_{18}O$	154	
11.216	11.100	11.278	0.12	0.09	—			
11.546	11.478	11.677	0.38	0.25	—			
11.797	11.728	11.847	0.07	0.09	*p*-Cymenene	$C_{10}H_{12}$	132	[131,141]
12.200	12.133	12.265	0.44	0.49	Terpinolene	$C_{10}H_{16}$	136	[143]
12.408	12.372	12.448	0.02	0.03	—			
12.675	12.562	12.823	4.44	4.30	*p*-Cymene	$C_{10}H_{14}$	134	[131,138,141,143–147,256]
12.916	12.837	13.003	1.93	1.68	Limonene	$C_{10}H_{16}$	136	[131,143–145,147,256]
13.058	13.003	13.148	0.95	0.98	Eucalyptol	$C_{10}H_{18}O$	154	[143,144,146,147,256]
13.293	13.200	13.402	4.37	4.45	2,2,6-Trimethylcyclohexanone	$C_9H_{16}O$	140	[130,131,138,141,144,145,148,149,256]
14.185	14.027	14.273	0.47	0.27	—	$C_{10}H_{20}O$	156	
14.421	14.387	14.470	0.03	0.04	—			
14.685	14.602	14.842	1.43	1.04	3-δ-Carene	$C_{10}H_{16}$	136	[130]
15.007	14.955	15.070	0.04	0.04	—	$C_{10}H_{18}O$	154	
15.227	15.143	15.358	0.12	0.08	—			
15.530	15.413	15.678	0.14	0.11	—			
15.990	15.920	16.053	0.17	0.17	—	$C_{10}H_{18}$ or $C_9H_{14}O$	138	
16.227	16.177	16.280	0.12	0.12	—			
16.351	16.295	16.400	0.18	0.22	Terpinolene	$C_{10}H_{16}$	136	[138,143,146]
16.501	16.447	16.573	0.15	0.13	Pinocarvone	$C_{10}H_{14}O$ or $C_{11}H_{18}$	150	[131,141,145,149,256]

Ret. Time	Start TM	End TM	% Area	% Height	Compound Name	Formula	Mol Weight	Bibliographic Similarity
16.599	16.575	16.647	0.03	0.04	—			
16.727	16.653	16.825	0.76	0.71	—			
17.139	17.090	17.202	0.04	0.05	α-Pinene oxide	$C_{10}H_{16}O$	152	[141,146]
17.337	17.267	17.573	0.39	0.17	---			
17.844	17.755	17.967	0.56	0.38	—			
18.031	17.978	18.098	0.12	0.12	Rose oxide	$C_{10}H_{18}O$	154	[141,144,147]
18.483	18.337	18.567	0.21	0.12	—	$C_{10}H_{18}O$	154	
18.707	18.633	18.800	0.35	0.31	Verbenone	$C_{10}H_{14}O$	150	[130,131,138,141,143–147,149]
18.874	18.828	18.935	0.04	0.04	—		154	
19.060	18.970	19.153	1.67	1.47	α-Campholenal	$C_{10}H_{16}O$	152	[141,147,256]
19.212	19.167	19.308	0.13	0.11	Verbenol	$C_{10}H_{16}O$	152	[130,149]
19.844	19.650	19.955	5.77	4.81	*trans*-Pinocarveol	$C_{10}H_{16}O$	152	[131,145–147,256]
20.011	19.965	20.137	0.19	0.12	—			
20.257	20.172	20.360	0.74	0.61	(L)-(-)-Camphor	$C_{10}H_{16}O$	152	[130,138,141,143,146,147,149]
20.583	20.498	20.660	0.23	0.21	—			
20.741	20.695	20.818	0.09	0.09	β-Linalool	$C_{10}H_{18}O$	154	[143,147,148]
21.177	21.047	21.232	0.55	0.42	Isopinocamphone	$C_{10}H_{16}O$	152	[143,145,147]
21.302	21.232	21.393	0.97	0.72	α-Pinocarvone	$C_{10}H_{14}O$	150	[143,145,149]
21.441	21.393	21.527	0.30	0.26	—			
21.939	21.790	22.010	1.88	1.54	Borneol (same as Camphol)	$C_{10}H_{18}O$	154	[130,131,138,141,143–149,256]
22.055	22.010	22.100	0.30	0.27	—			
22.171	22.100	22.308	1.22	1.02	*trans*-Pinanone	$C_{10}H_{16}O$	152	[143,147]
22.522	22.385	22.623	1.15	0.99	4-Terpineol	$C_{10}H_{18}O$	154	[138,141,145–147,256]
23.115	23.022	23.190	0.22	0.17	—			
23.513	23.415	23.560	0.80	0.68	(-)-Myrtenal	$C_{10}H_{14}O$	150	[145,147,256]
23.597	23.560	23.710	0.59	0.53	Myrtenol	$C_{10}H_{16}O$	152	[131,141,144–146,149,256]

Ret. Time	Start TM	End TM	% Area	% Height	Compound Name	Formula	Mol Weight	Bibliographic Similarity
23.995	23.892	24.067	0.11	0.08	—			
24.337	24.262	24.420	0.29	0.26	Verbenone	$C_{10}H_{14}O$	150	[130,131,138,141,143–147,149]
24.982	24.933	25.023	0.03	0.03	—			
25.118	25.060	25.163	0.04	0.04	—			
25.255	25.183	25.358	0.17	0.13	—	$C_{10}H_{16}O$	152	
25.658	25.562	25.765	0.44	0.32	Isobornyl acetate	$C_{12}H_{20}O_2$	196	[138,141,143,146–148]
25.924	25.872	25.992	0.04	0.04	*cis*-Carveol	$C_{10}H_{16}O$	152	[141,144,146,149]
26.449	26.302	26.550	0.79	0.50	—	$C_{10}H_{18}O$	154	
26.866	26.797	26.973	0.11	0.09	—	$C_{10}H_{14}O$ or $C_{11}H_{18}$	150	
27.238	27.212	27.283	0.02	0.02	*trans*-Pinocarveol	$C_{10}H_{16}O$	152	[144,146,256]
27.475	27.377	27.568	0.52	0.42	—			
27.843	27.802	27.917	0.03	0.03	—			
29.416	29.285	29.585	8.31	6.74	Bornyl acetate	$C_{12}H_{20}O_2$	196	[138,141,143,146–149,256]
29.999	29.947	30.033	0.03	0.04	Isobornyl acetate	$C_{12}H_{20}O_2$	196	Bornyl acetate exclusion
30.201	30.118	30.285	0.20	0.17	Myrtenyl acetate	$C_{12}H_{18}O_2$	194	[145–147]
30.730	30.690	30.802	0.04	0.04	—			
30.910	30.872	30.952	0.03	0.03	—	$C_{15}H_{24}O$	220	
31.086	31.020	31.150	0.10	0.10	—	$C_{12}H_{18}O_2$	194	
31.995	31.917	32.093	0.45	0.37	*trans*-Pinocarvyl acetate	$C_{12}H_{18}O_2$	194	Myrtenyl acetate exclusion
32.188	32.115	32.270	0.14	0.12	—			
32.778	32.698	32.850	0.17	0.15	Carvyl acetate	$C_{12}H_{18}O_2$	194	[147]
33.356	33.290	33.432	0.11	0.10	α-Cubebene	$C_{15}H_{24}$	204	[141]
34.608	34.433	34.718	0.92	0.56	(+)-Cyclosativene	$C_{15}H_{24}$	204	[138,147]
35.112	35.020	35.212	0.64	0.51	α-Copaene	$C_{15}H_{24}$	204	[141,145–147]
35.457	35.402	35.612	0.11	0.09	—			

Ret. Time	Start TM	End TM	% Area	% Height	Compound Name	Formula	Mol Weight	Bibliographic Similarity
36.018	35.948	36.100	0.07	0.06	(+)-Sativen	$C_{15}H_{24}$	204	Aromadendrene exclusion
36.465	36.423	36.533	0.04	0.04	—	$C_{15}H_{24}O$	220	
37.040	36.957	37.135	0.13	0.10	Patchoulene	$C_{15}H_{24}$	204	141
37.765	37.683	37.833	0.14	0.12	Caryophyllene	$C_{15}H_{24}$	204	141,143
37.983	37.937	38.037	0.02	0.02	(+)-Aromadendrene	$C_{15}H_{24}$	204	131,141,143,145–147,256
39.060	39.002	39.115	0.03	0.03	—	$C_{15}H_{24}$	204	
40.262	40.143	40.377	0.98	0.75	Alloaromadendrene	$C_{15}H_{24}$	204	131,141,143,145–147,256
41.113	41.060	41.183	0.04	0.04	*Epi*-Cubebol	$C_{15}H_{26}O$	222	141,146,147
41.341	41.282	41.413	0.10	0.09	—	$C_{15}H_{24}$	204	
42.048	41.968	42.130	0.17	0.14	β-Eudesmene	$C_{15}H_{24}$	204	143,145
42.248	42.177	42.348	0.31	0.24	Viridiflorene	$C_{15}H_{24}$	204	131,256
42.515	42.453	42.603	0.04	0.04	—			
42.828	42.765	42.907	0.10	0.09	α-Muurolene	$C_{15}H_{24}$	204	141,145,146
43.622	43.555	43.693	0.15	0.13	—			
44.009	43.925	44.095	0.46	0.35	δ-Cardinene	$C_{15}H_{24}$	204	131,138,141,145–147
44.175	44.117	44.283	0.17	0.13	—		202	
45.273	45.222	45.328	0.04	0.04	α-Calacorene	$C_{15}H_{20}$	200	141,146,147
46.865	46.790	46.932	0.11	0.09	(L)-Ledol	$C_{15}H_{26}O$	222	130,144,145,147,256
47.308	47.250	47.367	0.06	0.05	Spathulenol	$C_{15}H_{24}O$	220	131,141,145,147,149,256
47.508	47.432	47.603	0.14	0.11	Caryophyllene oxide	$C_{15}H_{24}O$	220	131,141,145,146,149
48.270	48.143	48.472	2.70	1.65	Viridiflorol	$C_{15}H_{26}O$	222	131,138,141,144–147,149,256
48.846	48.742	48.970	0.76	0.53	Epiglobulol	$C_{15}H_{26}O$	222	131,145,149
49.148	49.053	49.243	0.14	0.09	Copaborneol	$C_{15}H_{26}O$	222	256

Appendix C – ^{1}H NMR Analysis of Gingerols Extract – Fraction 1

Figure 31 shows the ^{1}H NMR graph of the gingerols for the existing sample. The data are also reported in Table 25, under the name of Fraction 1.

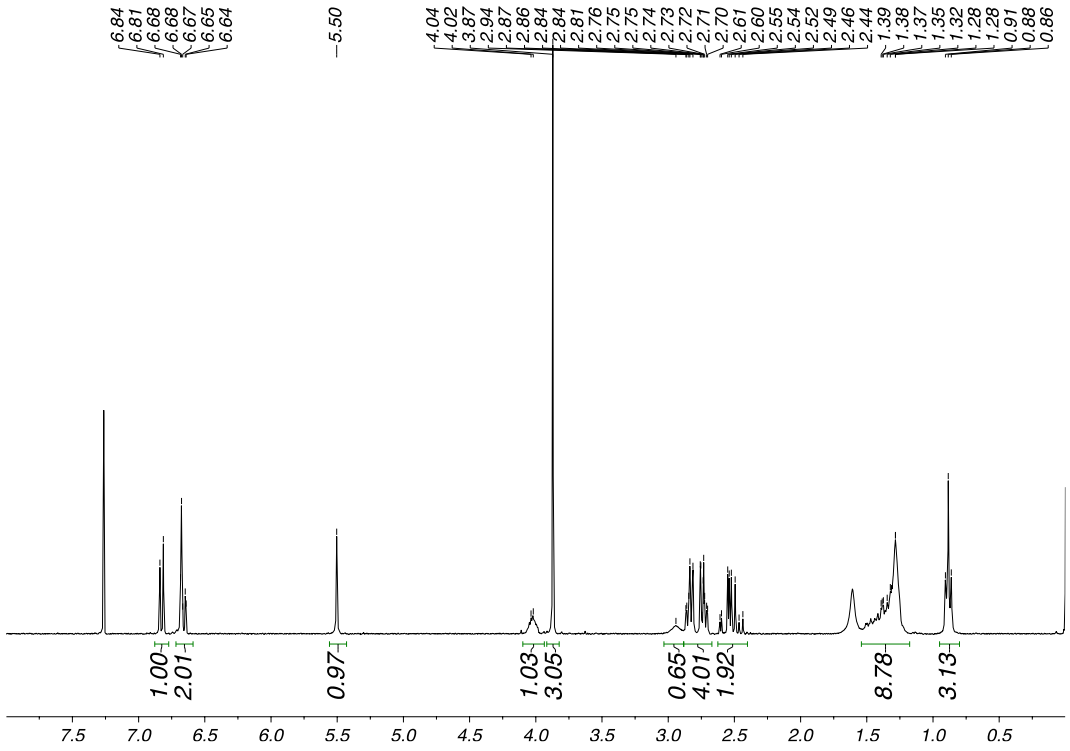

Figure 31 – ^{1}H NMR analysis of gingerols sample also identified as Fraction 1

Appendix D – β-Carotene Inhibition by Butylated Hydroxyanisole (BHA)

For the development of the assay, there must be a substance whose response to β-carotene is known. Thus, butylated hydroxyanisole (BHA) is used by standard. This antioxidant consists of a mixture of two isomeric organic compounds: 2-*tert*-butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole.

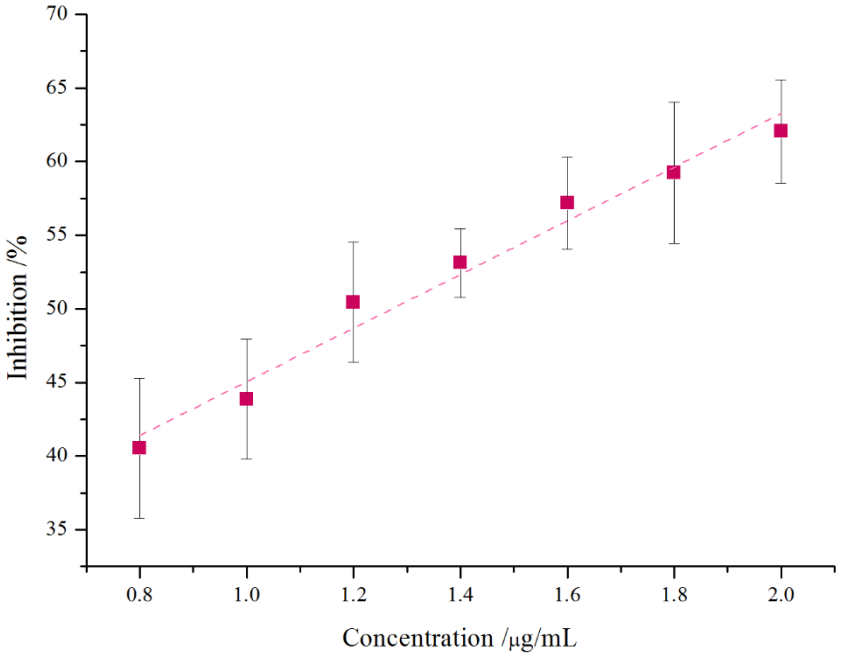

Figure 32 – Graphic of the inhibition of the BHA standard by the emulsion β-carotene and linoleic acid. Dashed lines represent the bleaching behaviour of BHA.

As the concentration of BHA increases, the percentage of inhibition also increases. It is possible to obtain an EC_{50} for the BHA, this being 1.26 ± 0.18 μg/mL.

Appendix E – β-Carotene Inhibition by γ-CD

Figure 33 shows the inhibition caused by γ-CD.

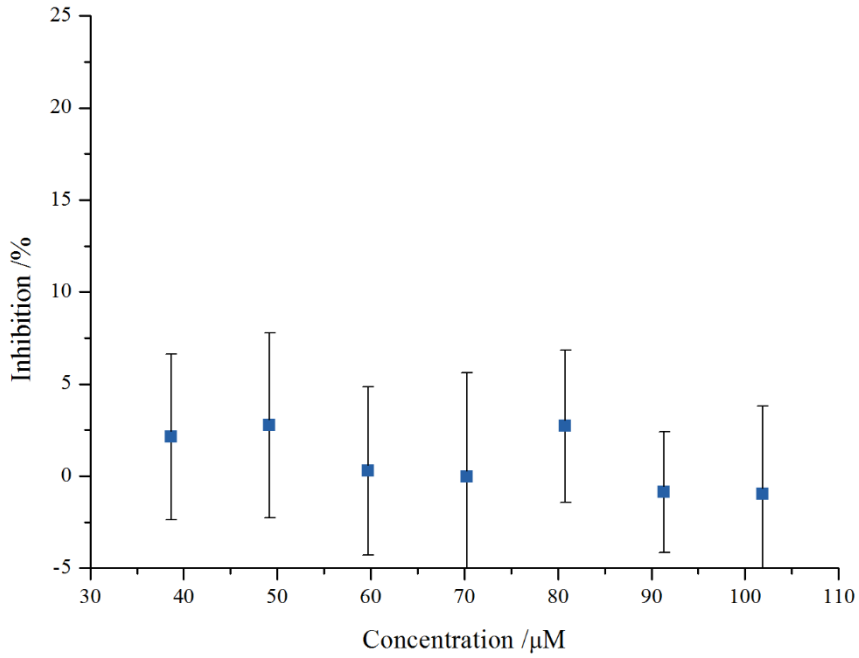

Figure 33 – Graph of the inhibition of γ-cyclodextrin by the emulsion of β-carotene and linoleic acid.

Appendix F – $ABTS^{\cdot+}$ Assay: Ascorbic Acid Inhibition Graphic

The ascorbic acid' absorbance data was applied in the following equation – Equation 5 –, in order to obtain the percentage inhibition.

$$\%\ \text{of inhibition} = \frac{ABS^{c=0} - ABS^{c=?}}{ABS^{c=0}} \times 100 \quad - \textit{Equation 5}$$

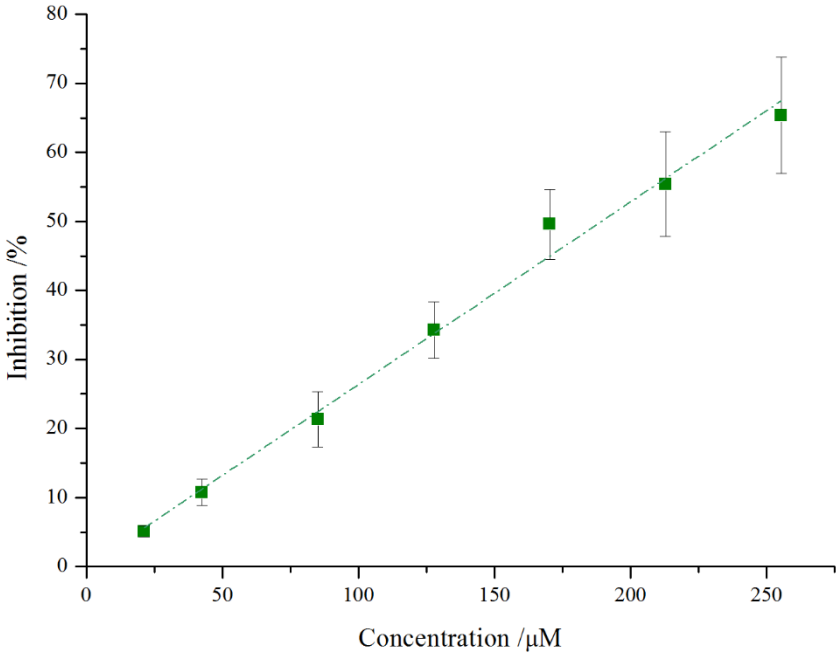

Figure 34 – Graph of the inhibition of ascorbic acid in $ABTS^{\cdot+}$.

As the concentration of ascorbic acid increases, the percentage of inhibition also increases. It is possible to obtain an EC_{50} for the acid ascorbic, this being 183.3 ± 14.9 µM.